# DNA Damage Signaling in Cells Coinfected with Adeno-Associated Virus and Herpes Simplex Virus Type 1

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Rebecca Vogel

aus

Deutschland

Promotionskomitee

Prof. Dr. Urs F. Greber

Prof. Dr. Michael O. Hottiger

Prof. Dr. Cornel Fraefel (Vorsitz und Leitung der Dissertation)

Zürich, 2013

# Table of content

# 1 Summary

The main goal of this PhD project was to contribute to our understanding of the molecular mechanisms of interaction between AAV2 and one of its helper viruses, HSV-1, and the host cell.

In the first part of the thesis I examined the localization and activation of DNA damage repair (DDR) proteins upon infection with HSV-1 alone or coinfection with AAV2 and HSV-1. I observed that consistent with previous reports the catalytic subunit (cs) of one of the main kinases in DDR, DNA-PK, was degraded in an HSV-1 ICP0-dependent manner. This degradation was observed also in cells coinfected with HSV-1 and AAV2, although it was markedly delayed. The delayed degradation of DNA-PKcs by AAV2 coinfection affected signaling to downstream substrates such as RPA32. In addition, p53 and Chk2 (targets of DNA-PK and ATM) were found activated in ATM-deficient cells only when coinfected with AAV2 and HSV-1 but not in ATM-deficient cells infected with HSV-1 alone.

During the AAV2 DNA replication process, DNA lesions (gaps) are created by the AAV2 Rep protein. The virus may therefore benefit from the activation of cellular DDR signaling because cellular repair factors are recruited into viral replication compartments (RCs). Indeed, I found several proteins of the cellular base excision repair (BER)/ single-strand break repair (SSBR) machinery in HSV-1 supported AAV2 RCs, including PARP1, XRCC1, RCF1, and ligase I.
I also investigated the influence of two different central elements of the DDR on AAV2 DNA replication. I found that while the MRN complex (the main sensor of ds breaks in cells) was not necessary to mediate activation of DDR signaling via RPA32, experiments in NBS1 negative cells indicated that the presence of a functional MRN complex or NBS1 clearly aids HSV-1-supported AAV2 DNA replication. In contrast, the presence of ATM (one of the three main kinase in DDR) clearly reduced HSV-1 supported AAV2 DNA replication, although the kinase was found activated and recruited into AAV2 RCs in coinfected cells.

In the last part of the thesis I set out to investigate how AAV2 can mediate the observed delay in the HSV-1 induced degradation of DNA-PKcs. To explore potential mechanisms responsible for this, I compared (i) levels, (ii) activity, and (iii) subcellular localization of ICP0 in coinfected cells and cells infected with HSV-1 alone, and I investigated a possible role of (iv) Ku70 and (v) AAV2 Rep 78/68 in the protection of DNA-PKcs from proteasomal degradation. The results can be summarized as follows: the levels of ICP0 were comparable and USP7, another target of ICP0-induced proteasomal degradation, was degraded equally efficient in cells infected with HSV-1 alone and in coinfected cells. Using recombinant HSV-1 expressing either cytosol- or nuclear-restricted ICP0 I observed that nuclear ICP0 was both

essential and sufficient to mediate degradation of DNA-PKcs. Ku70 is known to recruit DNA-PKcs to cellular DNA and localized to AAV2 RCs in coinfected cells. However, stabilization of DNA-PKcs and recruitment to RCs was Ku70-independent. AAV2 Rep 78/68 in absence of AAV2 DNA did not prevent HSV-1-mediated degradation of DNA-PKcs. Finally, I could also exclude AAV2 DNA as exclusive source of increased stabilization of DNA-PKcs in coinfected cells. On the basis of these results, I hypothesized that Rep 78/68 mediates recruitment of DNA-PKcs to sites of AAV2 DNA within AAV2 RCs, which further triggers phosphorylation of DNA-PKcs and stabilization.

In conclusion, I identified several factors of the cellular DNA sensing and repair machinery as components of HSV-1 and/or HSV-1 supported AAV2 RCs. In addition, I investigated the influence of two main players of the cellular DDR (NBS1 and ATM) on AAV2 DNA replication. The data contribute to a better understanding of the complex mechanisms of interaction between AAV2, its helper viruses, and the coinfected cell. Since AAV2 is a widely used vector in biomedical applications, the detailed knowledge of the activation and localization pattern of cellular proteins during AAV2 infection, may have important practical implications in the field of AAV2 vector research and gene therapy.

# 2 Zusammenfassung

Auf Grund seiner geringen genetischen Komplexität ist das Adeno-assoziierte Virus Typ 2 (AAV2) nicht nur abhängig von einer Wirtszelle, sondern auch von der Kopräsenz eines Helfervirus, wie zum Beispiel dem Herpes simplex Virus Typ 1 (HSV-1). In diesem Dissertationsprojekt hatte ich die Möglichkeit, die komplexe und faszinierende Interaktion der beiden Viren (AAV2 und HSV-1) und der Wirtszelle zu studieren. Das Hauptziel dieser Doktorarbeit war es, einen Beitrag zum Verständnis der molekularen Mechanismen der Interaktionen zwischen AAV2, HSV-1 sowie der koinfizierten Wirtszelle zu leisten.

Im ersten Teil der Arbeit untersuchte ich die Lokalisation und Aktivierung einer Auswahl von DNA Sensor- und Signaltransduktionsproteinen. Diese Proteine werden im Allgemeinen als „DNA damage response (DDR) factors“ bezeichnet. Im Einklang mit früheren Berichten beobachtete ich, dass eine der wichtigsten DDR Kinasen der Signaltransduktion nach Erkennung von DNA Schäden, DNA-PKcs, durch die Infektion mit dem HSV-1 Virus abgebaut wird. Dieser Abbau findet auch in Zellen statt, welche mit AAV2 und HSV-1 koinfiziert sind. In diesem Fall konnte jedoch eine deutliche Verzögerung dieses Prozesses beobachtet werden. Neben einem verzögerten Abbau von DNA-PKcs konnte auch dessen Phosphorylierung (an der Aminosäure Serin 2059) sowie Lokalisation in HSV-1-unterstützten AAV2 Replikationskompartimenten nachgewiesen werden. Die beobachtete Aminosäurephosphorylierung steht im Zusammenhang mit der Aktivierung der Kinaseaktivität des Enzymes. In der Tat konnte ich eine Auswirkung auf die Signaltransduktion von nachgeschalteten Substraten wie RPA32, p53 und Chk2 feststellen.

Eine Folge der Aktivierung der zellulären DDR Signalisierung in infizierten Zellen könnte die Rekrutierung von zellulären Reparaturfaktoren in virale Replikationszentren sein. Tatsächlich fand ich mehrere Proteine des zellulären Basenexzisionsreparatur (BER) bzw. des DNA Einzelstrangbruch-Reparatur (SSBR)-Mechanismus in AAV2 Replikationszentren wie z.B. PARP1, XRCC1, RCF1 und Ligase I. Während der AAV2 DNA-Replikation werden, für die DNA Replikation essentielle, DNA-Läsionen (gaps) von AAV2 Rep-Proteinen erzeugt. Um die Integrität des AAV2 Genoms zu wahren, müssen diese gaps wieder ligiert werden. Die identifizierten BER/SSBR Proteine könnten möglicherweise in diesem Zusammenhang eine zentrale Rolle für die AAV2 DNA Replikation und Aufrechterhaltung der Genomintegrität spielen.

Im Weiteren untersuchte ich den Einfluss von zwei verschiedenen zentralen Proteinen der Signaltransduktion auf die AAV2 DNA-Replikation. Ich konnte beobachten, dass in koinfizierten Zellen der MRN-Komplex (einer der Hauptsensoren von DNA Schäden) zwar nicht erforderlich für die Signaltransduktion zum RPA32 Protein ist, aber dass das Vorhandensein eines funktionellen MRN Komplex (oder

dessen Untereinheit NBS1) eine deutlich unterstützende Wirkung auf die AAV2 DNA-Replikation hat. Im Gegensatz dazu zeigte die Anwesenheit von ATM (neben DNA-PK und ATR die dritte Hauptkinase der DDR Signaltransduktion) eine deutlich hemmende Wirkung auf die HSV-1 unterstützte AAV2 Replikation.

Da ich eine Stabilisierung von DNA-PKcs in koinfizierten Zellen beobachten konnte, untersuchte ich im letzten Teil meiner Doktorarbeit mögliche Mechanismen des verzögerten Abbaus dieser Kinase. Es war bekannt, dass das HSV-1 Protein ICP0 den Abbau von DNA-PKcs induziert. Deshalb untersuchte ich (i) die Expression, (ii) die Aktivität und (iii) die subzelluläre Lokalisation von ICP0 in koinfizierten Zellen. Des Weiteren wurde eine mögliche Rolle (iv) des zellulären Ku70 und (v) des viralen Rep 78/68 Proteins (beides DNA-PKcs-Interaktionspartner) in der Stabilisierung von DNA-PKcs untersucht. Die Ergebnisse können wie folgt zusammengefasst werden: die Höhe der ICP0-Expression ist vergleichbar in HSV-1 infizierten und HSV-1 und AAV2 koinfizierten Zellen. Ein weiteres Protein welches in Anwesenheit von ICP0 in der Zelle degradiert wird, USP7, wurde auch in HSV-1 und AAV2 koinfizierten Zellen effizient degradiert. Unter Verwendung von rekombinanten HSV-1 Viren, die entweder cytoplasmatisch oder nukleär lokalisiertes ICP0 exprimieren, konnte ich beobachten, dass die nukleäre Form des ICP0 sowohl notwendig als auch ausreichend für den ICP0-vermittelten Abbau von DNA-PKcs ist. Ku70 ist dafür bekannt, dass es DNA-PKcs an zelluläre DNA-Läsionen rekrutiert und ebenso in AAV2 Replikationszentren nachzuweisen ist. Jedoch konnten ich in koinfizierten Zellen nicht nur eine Ku70 unabhängige Stabilisierung sondern auch Rekrutierung von DNA-PKcs in AAV2 Replikationszentren beobachten. Obwohl die AAV2 Rep 78 und 68 Proteine die Degradation von DNA-PKcs, in Abwesenheit der AAV2-DNA-Replikation und der Formation von viralen Replikationszentren, nicht verhindern konnten, ist zu vermuten, dass DNA-PKcs durch die Assoziation mit den AAV2 Rep 78/68 Proteinen auch in Abwesenheit von Ku70 in AAV2 Replikationszentren rekrutiert wird. In einem letzten Experiment konnte ich ausschließen, dass die durch die AAV2 DNA verursachte Phosphorylierung von DNA-PKcs an der Aminosäure Serin 2059 für die Stabilisierung der Kinase verantwortlich ist.

Auf der Grundlage dieser Ergebnisse stelle ich die Hypothese auf, dass die Rep 78/68 vermittelte Rekrutierung von DNA-PKcs an die AAV2 DNA innerhalb AAV2 Replikationszentren nicht nur die Phosphorylierung von DNA-PKcs initiiert, sondern auch einen geschützten Raum für DNA-PKcs gegen die HSV-1-ICP0-induzierte Degradation der Kinase schafft, was schließlich zur beobachteten Verzögerung des Abbaus der Kinase führt.

Insgesamt konnte ich in dieser Arbeit mehrere Faktoren des zellulären Sensor-und Reparaturapparates von DNA-Läsionen als Komponenten von HSV-1 und/oder HSV-1 unterstützten AAV2 Replikationszentren definieren. Darüber hinaus untersuchte ich den Einfluss zweier Hauptakteure des zellulären DDR (NBS1 und ATM) auf die AAV2 DNA-Replikation. Diese Daten tragen zu einem besseren Verständnis der komplexen Mechanismen der Interaktion zwischen AAV2, seinem Helfervirus HSV-1 und der koinfizierten Wirtszelle bei. Da AAV2 ein weit verbreiteter Vektor in biomedizinischen

Anwendungen ist, kann das detaillierte Wissen über die Aktivierung und Lokalisation von zellulären Proteinen während der AAV2 Infektion einen wichtigen Beitrag leisten, um die Anwendung von AAV2 als Vektor für die Gentherapie weiter voran zu treiben.

# 3 Introduction

## 3.1 Viruses: origin, diversity and classification

The word virus comes from Latin and means toxin or poison. At the end of the 19th century, the Russian scientist D. Ivanovsky (67), the Dutch scientist M. Beijernick (208), and the German scientists F. Loeffler and P. Frosch (109) discovered pathogenic agents smaller than any known bacterium. M. Beijernick and F. Loeffler/P. Frosch could further show that these pathogens are the causative agents of tabacco mosaic disease and foot-and-mouth disease, respectively (109, 208). In general, viruses are infectious, obligate intracellular parasites (52). Within a susceptible host cell, the virus replicates and new virions (progeny infectious virus particles) are released (52).

Because they infect all three domains of life, Archaea, Bacteria and Eukarya, it is suggested that viruses originated very early in the evolution of life (54). Three different theories of virus evolution are under debate, the “virus first theory”, the “virus escape theory”, and the “virus reduction theory” (54). The “virus first theory” implicates an independent emergence of viruses from the last universal common ancestor (LUCA) of all known life forms (54). In this case viruses may have emerged in a non-cellular context, in a world of competing proteins and nucleic acids (54). However, the hypothesis of a LUCA without membrane is reviewed as contradictory and, therefore, the “virus escape theory” and the “virus reduction theory” may be more likely (54). The “virus escape theory” suggests that viruses have derived from fragmented genomes of ancestral RNA cells and have become autonomous and infectious RNA elements (54). The “virus reduction theory” suggests that a small RNA cell has become an endosymbiont of a larger RNA cell by losing its own translation machinery, becoming infectious and conserving the capability to replicate autonomously (54). While RNA viruses may have originated by escape or reduction from RNA cells, DNA viruses might have evolved directly from RNA viruses (54). In this context, it is suggested that genes encoding enzymes required for RNA-DNA-transition first appeared in viral genomes (54).
Out of these ancient viruses numerous different viruses evolved. One method to classify all of the different virus types is the Baltimore classification (first defined in 1971), which places viruses into one of seven groups depending on the method of viral mRNA synthesis (13). DNA and RNA viruses can have a double-stranded (ds) or single-stranded (ss) genome; however most of the DNA viruses contain ds genomes (e.g. polyoma-, adeno-, herpesviruses), while most of the RNA viruses contain ss genomes (e.g. retro-, flavi-, orthomyxoviruses; (52)). ss RNA viruses can be further divided into positive stranded RNA viruses (e.g. picornvirus), whose genomes can be directly transcribed by the cellular translation machinery, or negative stranded RNA viruses (e.g. paramyxovirus) which first have to convert their genomes into positive stranded RNA (by RNA-dependent RNA polymerase) before translation can occur (52). Positive and negative stranded viral ss DNA is not a feature of different categories of DNA viruses, but the replication product of linear ss DNA

genomes of e.g. parvoviruses, as both positive and negative sense viral genomes are packaged into infectious virions (52). Another classification system of viruses was developed by the International Committee on Taxonomy of Viruses. The classification system is organized in a branching hierarchy of the following viral taxa: Species, genus (-*virus*), sub-family (-*virinae*), family (-*viridae*), and order (-*virales*) (77). The members of each taxa share some significant properties including host range, cell and tissue tropism, pathogenicity, or vector specificity (77). In addition, the degree of relatedness of their genomes or genes is an important aspect for classification (77).

## 3.2 Adeno-associated virus 2 (AAV2)

### 3.2.1 Classification and pathogenesis.

Adeno-associated virus 2 (AAV2) is classified as a *Dependovirus* due to its replication deficiency in the absence of a helper virus (69). The genus *Dependovirus* belongs to the subfamily of *Parvovirinae (77).* Due to the ss nature of the viral genome, the family of *Parvoviridae* belongs to the *group II* viruses, according to the Baltomore classification system (13).

AAV2 has first been detected in electron microphotographs of simian adenovirus preparations (10, 112). Until now, over 100 AAV2 variants from different animal species have been isolated (58). The different serotypes display diverse genome and capsid structures as well as tissue tropisms, including host receptor usage and intracellular processing (58).

In humans, different AAV2 serotypes can infect various tissues (58) without causing any known pathology (179). The seroprevalence of AAV2 in the human population is more than 50% (179).

### 3.2.2 AAV2 structure and genome organization.

AAV2 is a small, non-enveloped virus with an icosahedral capsid of roughly 20 nm in diameter (Fig. 1A; (190)). The capsid is composed of 60 copies of three different structural proteins, VP1, VP2, and VP3, in a 1:1:10 ratio, protecting a linear, ss DNA genome of approximately 4.7-kb (190). AAV2 has a very simple genomic organization. The central part of the genome consists of two open reading frames (ORF), carrying the *rep* and *cap* genes (Fig. 1B, (190)). However, different promoters, alternative splicing, and non-conventional translational start codons give rise to 8 different AAV2 proteins. The overlapping, in-frame coding regions within the *rep* ORF are transcribed form two different promoters, p5 and p19, generating mRNAs which encode Rep 78 and Rep 52, respectively; alternative spliced mRNAs from these promoters encode Rep 68 and Rep 40 (Fig. 1B). Alternative splicing plays also an important role in the generation of the two different RNAs generated from the p40 promoter, encoding the AAV2 capsid proteins VP1 (2.4-kb mRNA, minor splicing product) as well as VP2 and VP3 (2.3-kb mRNA, main splicing product, Fig. 1B; (141)). Subsequently, the use of two different start codons on the 2.3-kb mRNA,

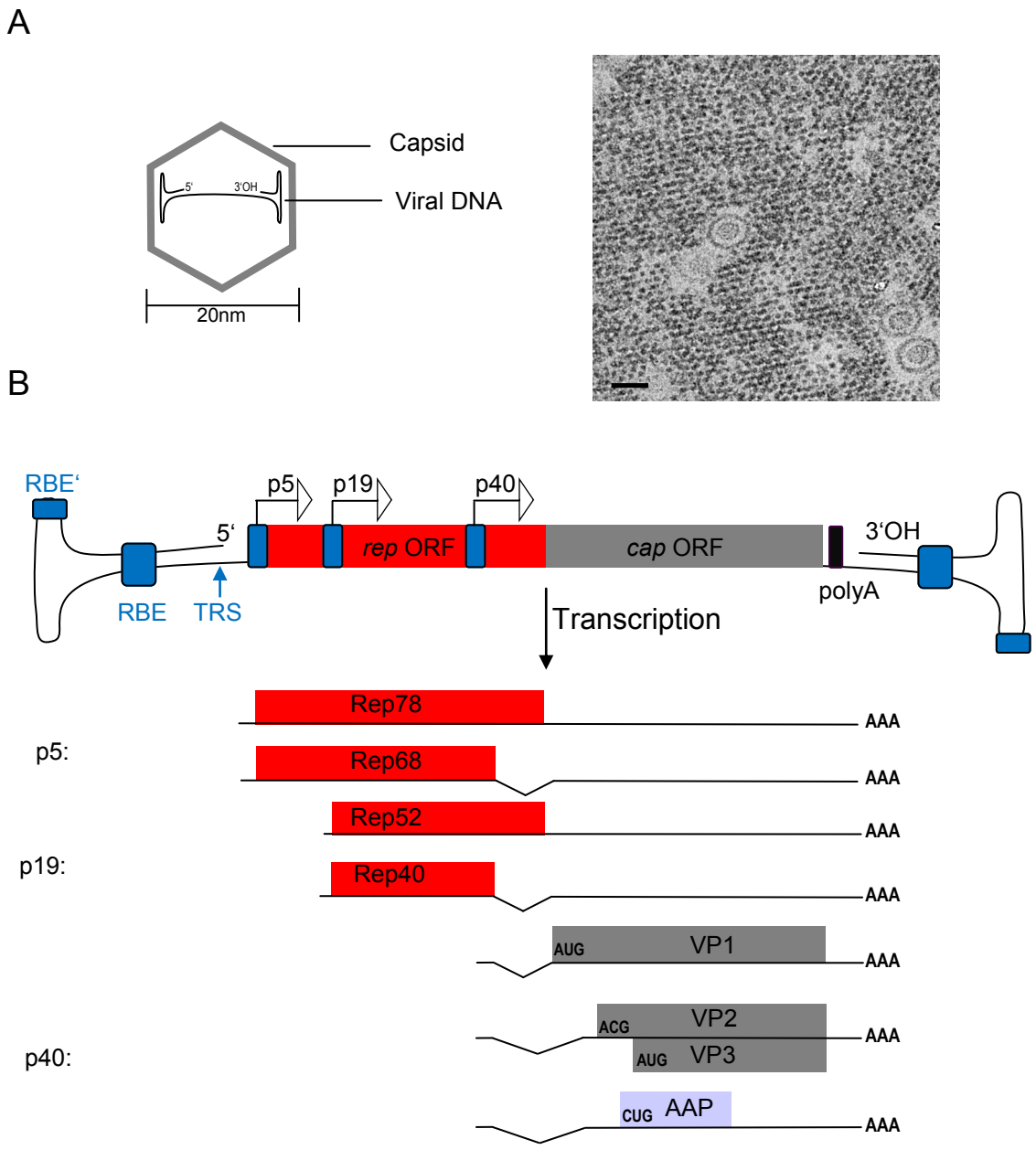



**Fig 1.** Structure of the AAV2 virion, genome, and transcripts. **(A)** The AAV2 ss DNA genome is enclosed by an icosahedral capsid of 20nm in diameter. Negative contrast electron photomicrograph (Elisabeth Schraner, University of Zürich). Scale bar 0.1 µm. **(B)** The AAV2 genome contains two open reading frames (ORF) *rep* and *cap* and is flanked by inverted terminal repeats (ITR) containing the RBE (GAGCGAGCGAGCGCGC), the RBE′ (CTTG), and the TRS (GTTGG) signals. The two ORFs *rep* and *cap* encode the non-structural Rep proteins (Rep40, Rep52, Rep68 and Rep72) and the capsid proteins (VP1, VP2, and VP3), transcribed by the p5 and p19 or p40 promoter, respectively. Transcription is terminated at the polyA signal, creating a polyA tail (AAA) in all transcripts. A minor group of spliced products contains the first translation initiation codon (AUG) for VP1. VP2 and VP3 are translated from two different start codons (ACG and AUG) of the main spliced product. This transcript encodes also the recently identified assembly activating protein (AAP), translated from a nonconventional CUG translation initiation codon of a nested, alternative ORF.

gives rise to both VP2 and VP3 (Fig. 1B; (18, 119)). More recently, a new AAV2 protein was identified and designated as assembly-activating protein (AAP; (162)). This protein is also encoded by the 2.3-kb mRNA of the cap gene and translated from a nonconventional CUG translation initiation codon of a nested, alternative ORF (Fig. 1B; (162)). The four non-structural, multifunctional Rep proteins provide functions necessary for regulation of AAV2 promoters (72, 96, 97, 137), genome replication (71, 73), packaging of viral genomes into capsids (36, 91, 118, 167), and rescue of the integrated AAV2 genome (21, 181). More precisely, Rep 52 and Rep 40 display ATPase enzymatic activity and are involved in the generation of ss viral genomes from ds replicative intermediates and subsequent encapsidation (36). Rep 78 and Rep 68 proteins have a high degree of homology concerning sequence and function. Both possess endonuclease, helicase, ATPase, and possibly ligase activities (121) and are important for AAV2 DNA replication, site-specific integration, and transcriptional regulation (71–73, 96, 97, 137). In addition, Rep 78/68 enhance or suppress AAV2 gene expression in presence or absence of a helper virus, respectively (137). The three colinear structural virus proteins VP1, VP2, and VP3 together form the AAV2 capsid (190). It has been shown that VP1 contains a conserved phospholipase activity that appears to be essential for infectivity of AAV2 (62). While VP1 and VP3 are essential for the production of intact virions, VP2 was shown to be dispensable for efficient packaging and infectivity and even tolerate large ligand-insertions useful e.g. to retarget AAV2 vectors to alternative receptors in gene therapy applications (185). The fourth, newly discovered gene product of the *cap* ORF, AAP, was shown to be essential for the assembly of the capsid subunits and the reproduction of AAV2 (162).

The AAV2 *rep* and *cap* ORFs are flanked by hairpin structured, self-priming inverted terminal repeats (ITRs) of 145 nucleotides (Fig. 1B; (24)). These essential multipalindromic DNA termini contain all cis-acting elements required for replication, packaging, and site specific integration events (190). The important sequence elements of the ITRs are the Rep binding element (RBE), a small palindrome that comprises a single tip of an internal hairpin (RBE'), and the terminal resolution site, TRS, located 13 nucleotides upstream of RBS (Fig. 1B; (190); discussed in more detail below).

### 3.2.3 AAV2 entry.

Previous experiments showed that replication of AAV2 in cell culture was only successful when AAV2 was inoculated simultaneously with adenoviruses (Ad; (10)), leading to the suggestion that these Ad-associated particles behave as defective viruses (10). Today, it is well known that AAV2 has a unique biphasic life cycle, determined by the permissiveness of the cells and the presence of a helper virus.

Although AAV2 entry does not depend on a helper virus, it was shown that it is influenced by the presence of a helper virus (203). AAV2 can infect dividing as well as non-dividing and terminally differentiated cells (23, 45, 202). The virus is internalized by the formation of clathrin-coated pits after receptor binding (15). Heparan sulfate proteoglycans (HSPGs, C. (165)) as well as the coreceptors αvβ5 integrin (165), α5β1 integrin (9), fibroblast growth factor receptor (FGFR1, (140)), and

hepatocyte growth factor receptor (c-met; (86)) were identified as receptors for AAV2 entry.
A recent study showed that following uptake, AAV2 exploits the microtubule network for rapid cytoplasmic trafficking in endosomal compartments, unidirectional towards the perinuclear region, where viral escape is mediated by endosomal acidification (201). However, crucial cellular factors involved in trafficking as well as viral uncoating and nuclear entry are not yet characterized. It has been suggested that viral uncoating occurs before or during nuclear entry (203). In the presence of Ad, however, nuclear translocation of intact AAV2 capsid into the nucleus was observed (203).

### 3.2.4 AAV2 latency.

In the absence of a helper virus, AAV2 establishes latent infection. During latency, the AAV2 genome is maintained in an episomal state in the cell nucleus or integrated preferentially into a specific site (19q13.3-qter or AAVS1) on human chromosome 19 (95, 148), but integration into other host chromosomes have also been reported (76). In addition, there is indication that integration occurs adjacent to accessible open chromatin regions. The observed site specific integration of AAV2 in human cell lines occurs in 70–95% of events (94, 95, 148). However, in latently-infected human tissues the majority of wtAAV2 genomes were detected in form of circular episomes (2); therefore, the significance of site-specific integration of wtAAV2 in natural infection remains elusive. In case of AAV2 integration, the proviral structures of AAV2 integrants are typically arranged in head-to-tail concatemers, with microsequence homology at the viral–cellular junctions (191). Both the ITRs and the AAV2 Rep 78/68 proteins are essential elements for AAV2 integration (191). Rep is responsible for targeting integration into AAVS1 by forming a bridge between viral ITRs and the target site in AAVS1 Specifically, Rep 78/68 proteins recognize the RBE sequence motifs within the AAV2 and the cellular genome (42). Besides the site-specific binding activity, also the endonuclease activity was shown to be required for site-specific integration of the AAV2 genome (190). In contrast, a functional TRS and replication of the donor ITR may not be a prerequisite for AAV2 integration (191). Another component of the AAV2 genome implicated in site-specific integration is the p5 promoter (138). It was shown that the p5 promoter functions as a primary target sequence for Rep upon site-specific integration (138). In this context, it is suggested that the p5 promoter is a key component to regulate lytic vs latent infection by conversion from a transcription initiation complex into a structure that facilitates efficient Rep-dependent integration (68). Although the data is inconsistent, the cellular non-homologous end joining (NHEJ) machinery (described in more detail below) may influence AAV2 integration events (113). In cell culture experiments, one of the main factors of NHEJ, the catalytic subunit of DNA dependent protein kinase (DNA-PKcs), was shown to enhance integration of both ss rAAV2 and ds rAAV2 DNA (31). Another report showed that in particular the NHEJ protein ligase IV supports site-specific integration of incoming ss AAV2 vectors (43). Another factor implicated in NHEJ mediated V(D)J recombination events is the high mobility group protein 1 (HMG1; (151)). This protein was shown to bind to Rep 78 and enhance both RBS

binding and nicking activities of Rep, thereby also supporting site-specific integration of the AAV2 genome (41). The occurrence of variable deletions of the ITR and chromosome sequences at the junction regions between host and viral DNA supports the model of recombination mediated AAV2 integration (191). Although this data suggests a supportive role of the NHEJ machinery on AAV2 integration, it has to be mentioned also that in an animal experiment, rAAV2 genome integration was shown to be strongly enhanced in DNA-PKcs-deficient SCID mice (161). The reason for the discrepancy of the function of DNA-PKcs in the different experiments is not clear.

### 3.2.5 AAV2 lytic infection.

In presence of a helper virus, latent AAV2 genomes can be rescued and enter a lytic replication cycle (190). Besides Ad and herpesviruses (190), also other viruses, including papillomavirus (16, 30, 178), can provide helper functions for AAV2 infection. Upon lytic infection, AAV2 DNA replication occurs at discrete sites in the nucleus, so-called replication compartments or replication centers (RCs). Several small RCs rapidly expand and fuse to large structures, which suspend the cellular chromatin and fill the entire nucleus (55, 70, 75, 164, 188). AAV2 DNA replication occurs as a self-priming, single-strand displacement mechanism (168).The initial step of AAV2 replication is the conversion of the incoming viral ss DNA into a ds DNA (190). This step is believed to be mediated through the host replication machinery; besides the AAV2 Rep 78/68 proteins also helper virus factors may be implicated (190). The complementary self-annealing sequence of the AAV2 ITR is the basis for initial second-strand synthesis, by providing a base-paired 3’ hydroxyl group primer for unidirectional DNA synthesis (190). By providing its own DNA primer, AAV2 DNA replication exclusively uses leading-strand DNA synthesis (20). Although both helper viruses, Ad and HSV-1, express their own polymerases for viral DNA replication, AAV2 seems to use only the HSV-1 polymerase (6), while the Ad DNA polymerase is not used for AAV2 replication (80, 144). Polymerase delta was found in AAV2 RCs (123) and therefore might be the cellular polymerase for Ad supported AAV2 DNA replication. Interestingly, polymerase delta was also found in HSV-1 supported AAV2 RCs (128). Although the HSV-1 polymerase complex was shown to be an important, albeit not essential, helper virus factor, the cellular polymerase may also be implicated in HSV-1 supported AAV2 replication. Second-strand synthesis of the ss AAV2 genome leads to the formation of a duplex DNA molecule that is covalently closed at one end by a hairpin structure. In order to complete replication of the viral genome, in a next step, called terminal resolution, the remaining hairpin has to be resolved and replicated to produce a linear double-stranded DNA (ds DNA, (190)). The AAV2 Rep 78/68 proteins initiate terminal resolution by binding to the RBS motif within the ITR and cleavage of one DNA strand at the TRS (190). The regenerated free 3’hydroxyl-group then provides the primer for the replication through the viral ITR, thereby maintaining the integrity of the 5′ terminus of the AAV2 DNA (190). In contrast to biding to the RBE, binding of Rep to the RBE′ at the tip of the ITR hairpin seems to be required for strand unwinding during terminal resolution but not for cleavage (28). In a last step called reinitiation, a double-hairpinned intermediate is formed by denaturation and reannealing of the linear ITR (190). This last step

initiates a new round of strand displacement synthesis generating a single-stranded AAV2 genome that can be packaged into a capsid (190).

3.2.6 The p5 promoter: an alternative origin of AAV2 DNA replication.
Besides its function in transcription and site-specific integration (see above), the p5 promoter can also serve as a Rep-dependent origin of DNA replication (56, 133, 172, 182). Upon HSV-1 coinfection, it was demonstrated that the RBS and TRS, present within the p5 promoter, function as a Rep- and HSV-1-dependent origin of AAV2 DNA replication (64). Further, replication from the two alternative AAV2 replication origins, p5 and ITR, was shown to occur simultaneously and independently (64). The distinct p5 and ITR RCs were juxtaposed to each other (64).

3.2.7 Cellular proteins supporting AAV2 replication in absence of a helper virus.
Cells exposed to a variety of genotoxic agents (e.g. HU or IR) can support wtAAV2 replication even in absence of a helper virus (204–206), suggesting that cellular factors of the DNA damage sensing and repair machinery are involved in AAV2 replication steps. A more recent study showed that although significant helper-independent AAV2 *rep* expression can be observed in cells exposed to genotoxic agents, only low levels of AAV2 DNA replication occurs(129). It is suggested that the interference of AAV2 with the cellular DDR and repair machinery promotes second-strand synthesis of the AAV2 genome to allow AAV2 gene expression (32, 50, 51, 129, 146). Two different groups examined the requirement for helper virus-independent AAV2 DNA replication in *in vitro* systems. In absence of a helper virus, the AAV2 Rep 78 or 68 proteins as well as the cellular replication factors proliferating-cell-nuclear-antigen (PCNA), polymerase delta, mini-chromosome maintenance (MCM) proteins, and replication factor C (RFC) were shown to be required and sufficient to reconstitute efficient AAV2 DNA replication in the *in vitro* replication system (122, 132). These cellular proteins can be substituted completely by the HSV-1 helicase-primase complex (UL5, UL8, and UL52), the HSV-1 major ss DNA binding protein ICP8 (UL29), and the HSV-1 polymerase complex (UL30 and UL42) to initiate replication on duplex DNA containing the AAV2 origins of replication in vitro (184).

## 3.3 Herpes simplex virus type 1 (HSV-1)

3.3.1 Classification and pathogenesis.
Herpes simplex virus type 1 (HSV-1), also referred to as human herpesvirus 1 (HHV-1), belongs to the genus *Simplexvirus* in the subfamily of the *Alphaherpesvirinae (77).* Viruses of the family of *Herpesviridae* have a ds DNA genome and, therefore, belong to the *group I* viruses according to the Baltimore classification system (13). HSV-1 is a common human neurotropic pathogen, with a worldwide seroprevalence

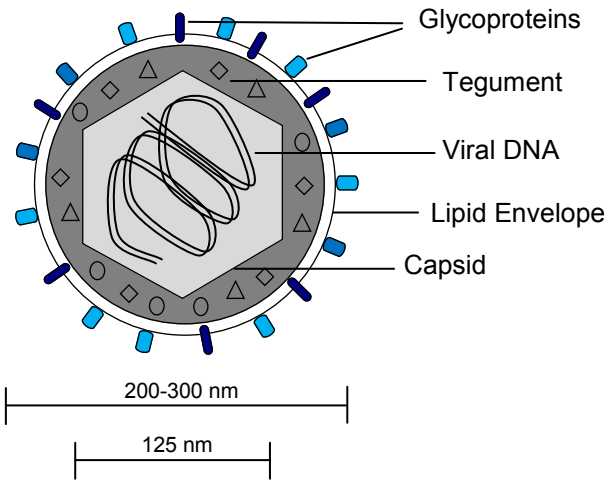
of up to 80% in adults (5). The virus causes lytic infection at the site of host entry and life-long latent infection in the proximal trigeminal ganglia (170). Hallmarks of the lasting infection are recurrent lesions with shedding of virions (52). However, also upon latent infection, hosts are still capable of spreading infection to other humans via asymptomatic shedding of virions (5). Most commonly, HSV-1 lytic infection takes place in orofacial mucosal epithelia cells, causing cold sore or fever blisters, but also genital mucosa epithelia cells (98) or cutaneous sites, such as fingers, toes or knees (134) can become infected. In some cases HSV-1 infection can cause severe meningo-encephalitis and kerato-conjunctivitis that can lead to permanent neurological damage with high mortality or corneal blindness, respectively (207). Genital herpes infections can also result in mother-to-infant transmission (163). HSV-1 infection of newborns can cause lesions of the skin, eyes, mouth, or encephalitis (163). In addition, HSV-1 infection of the preterm infant seems to be associated with lethargy, hypotension, temperature instability, and elevated aspartate aminotransferase levels (163).

### 3.3.2 HSV-1 structure and genome organization.

The HSV-1 virion has a diameter of approximately 300 nm and consists of four distinct compartments: the envelope, the tegument, the capsid, and the enclosed viral linear ds DNA genome of 152-kbp (Fig. 2A; (52)). The viral envelope is derived from host membranes and contains 11 viral glycoproteins, some of which play an important role in viral entry (134). The tegument is a thick layer of virus-encoded proteins involved in initial steps of HSV-1 infection, including trafficking to the nucleus, inhibition of cellular gene expression, and initiation of viral gene expression (5). The HSV-1 capsid is 160 Å thick and has a diameter of 1,25 Å (139). HSV-1 proteins, expressed form the genes UL18, UL19, UL26, UL26.5, UL35, UL38, and UL6 constitute the icosahedral capsid (74). More precisely, the major capsid protein VP5 and VP26 (UL19 and UL35) make up 162 capsomers (150 hexons and 12 pentons), which are connected by 320 triplexes of VP19C (UL18) and VP23 (UL38; (125, 209)). HSV-1 contain also large amounts of VP22a protein (UL26.5) and smaller amounts of VP24 and VP21 proteins (both expressed from UL 26), which are involved in the formation and processing of the capsid scaffold (74). The scaffolding proteins form a core (the scaffold) and interact with VP5 to support capsid assembly (74). In addition, the HSV-1 capsid shell also contains trace amounts of a few other proteins, including products of the UL6, UL12.5, UL15 and UL25 genes (74).

The HSV-1 capsid protects one copy of a tightly condensed viral ds DNA, free of bound histones or other proteins (74). The genome consists of two covalently linked fragments, designated long (L) and short (S) segments (52). Each segment is flanked by inverted repeats (155). The junction regions between the segments are arranged as follow: ab-UL-b′a′c′-US-ca (Fig. 2A; (192)). HSV-1 encodes about 89 proteins (134), approximately half of which are essential for virus replication in cell culture (Fig. 2A; (3)). Each HSV-1 gene promoter has a TATA box homology upstream of the start site of transcription (52), allowing viral gene expression by the cellar RNA polymerase II (52). To facilitate viral DNA replication, two replication origins are present in the repeated c region (OriS) and a third replication origin is located in the

A



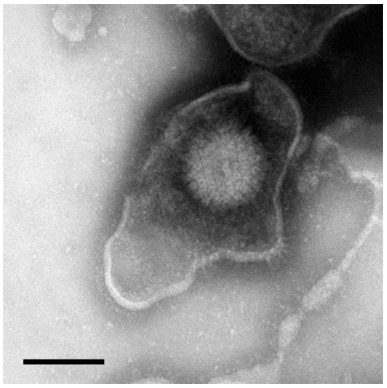
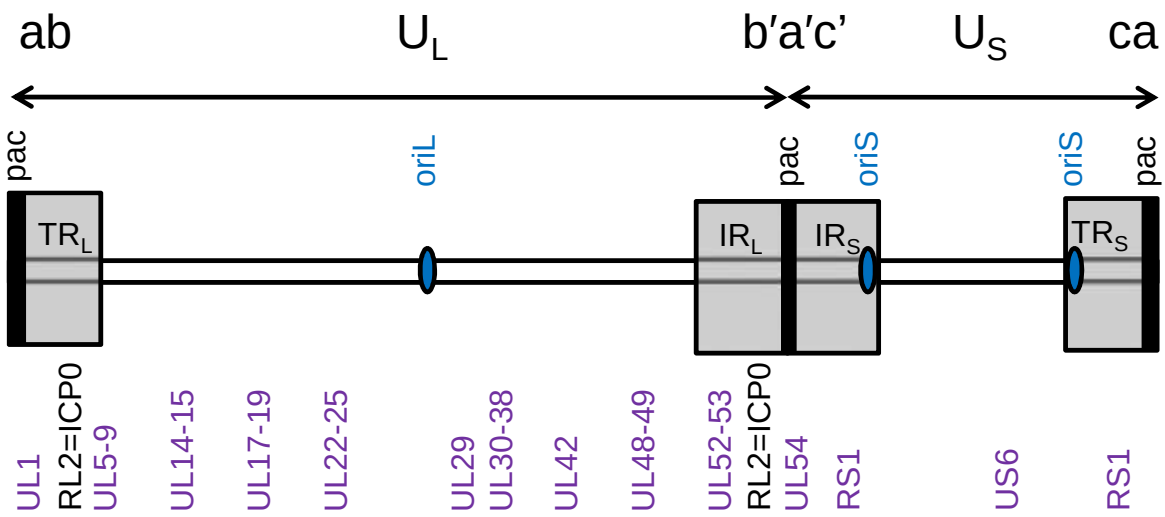



**Fig 2. (A)** Structure of the HSV-1 virion. The HSV-1 virion is composed of a lipoproteic envelope containing viral glycoproteins, the tegument, the icosahedral capsid, and the enclosed viral linear ds DNA genome of 152 kbp. The diameter of the enveloped HSV-1 particle ranges from 200-300 nm. The capsid has a diameter of 125 nm. Negative contrast electron photomicrograph (Elisabeth Schraner, University of Zürich). Scale bar 0.1µm.




essential genes

UL1 (glycoprotein L)
UL5 (comp. of helicase-primase complex)
UL6 (portal protein UL6)
UL7
UL8 (comp. of helicase-primase complex)
UL9 (origin binding protein UL9)
UL14 (tegument protein UL14)
UL15 (tripartite terminase subunit)
UL17 (virion-packaging protein Ul17)
UL18 (triplex capsid protein VP23)
UL19 (major capsid protein VP5)
UL22 (glycoprotein H)
UL23 (thymidine kinase)
UL24
UL25 (virion-packaging protein UL25)
UL29 (ICP8)
UL30 (DNA polymerase catalytic subunit)
UL31 (nuclear egress lamina protein UL31)
UL32 (DNA packaging protein UL32)
UL33 (tripartite terminase subunit)
UL34 (virion egress protein UL34)
UL35 (capsid protein VP26)
UL36 (tegument protein VP1/2)
UL37 (tegument protein UL37)
UL38
UL42 (DNA polymerase processivity factor)
UL48 (tegument protein VP16)
UL49 (tegument protein VP22 )
UL52 (comp. of helicase-primase complex)
UL53 (glycoprotein K)
UL54 (ICP27)
RS1=ICP4
US6 (glycoprotein D)

**Fig 2. (B)** Structure of the linear ds HSV-1 genome and schematic map of the essential HSV-1 genes (purple) and ICP0. The genome consists of two covalently linked segments: unique long (UL) and unique short (US). Each segment is flanked by inverted repeats TRL/IRL and TRS/IRS, respectively. ab-UL-b′a′c′-US-ca displays the arrangement of the regions. The genome conatins three origins of DNA replication (ori). Two OriS are present in the repeated c region of US, while OriL is located in the UL segment of the genome. Three DNA cleavage/packaging signals (pac) are present, flanking both termini of the genome and at the junction between the long and the short segments.

UL segment of the genome (OriL; (192)). Both, OriL and OriS contain A/T-rich regions, flanked by two recognition sites for the HSV-1 origin-binding proteins (OBPI, OBPII, and UL9; (192)). For packaging of the viral genome, three identical DNA cleavage/packaging signals (pac) are present at the termini of the genome as well as at the junction between the long and the short segments (52).

### 3.3.3 HSV-1 entry.

Several viral envelope glycoproteins are implicated in the HSV-1 entry process, including: gB, gC, gD, and the heterodimer gH/gL (5). The initial interaction between the virus and the host membrane occurs via binding of gC and/or gB to heparan sulfate proteoglycans (HSPGs; (5)). It is suggested that the enrichment of HSPGs at cellular protrusions (filopodia) may promote the initial attachment of HSV-1 to the host cell (5). The subsequent process of penetration depends on the cell type and the mode of entry (5) and can occur via direct fusion of the HSV-1 envelope with the plasma membrane (5) or via fusion with the membrane of an intracellular vesicle, after endosomal or phagosomal uptake of the virus (40, 60, 126–128). Both fusions with the plasma membrane and endocytic uptake can be pH-dependent or pH-independent (5). Although it was shown that the expression of receptors, nectin-1 and paired immunoglobulin-like type 2 receptor α (PILRα), correlates with endocytic uptake or virus-cell membrane fusion, respectively, another report showed that the same receptor can also mediate different modes of entry, depending on the cell (5). Nevertheless, fusion with both the cellular and vesicular membrane (after endocytic uptake), requires a multi-glycoprotein complex (gB, gD, and gH/gL; (5)) and one of the following cellular receptors: nectin-1 or -2 (in neurons, keratinocytes and epithelial cells), herpesvirus entry mediator (HVEM, in fibroblasts and ocular cell types), or 3-O sulfated heparan sulfate (3-O HS, in corneal fibroblasts; (124)). Binding of gD to one of these receptors leads to a conformational change of gD and further triggers gB to insert its fusion loops into the target cell membrane, allowing membrane fusion to take place (124). Fusion of the HSV-1 envelope with the cell membrane leads to the release of the nucleocapsid and tegument proteins into the host cytoplasm (5). For transport towards the nuclear membrane HSV-1 nucleocapsids bind dynein to shuttle along microtubules (160). As mentioned above, HSV-1 can enter the cell by receptor-mediated fusion between virus- and cell-membrane or receptor-mediated endocytosis (5). In neurons, HSV-1 entry seems to occur via direct fusion at the plasma membrane (5). The HSV-1 capsids are then transported retrogradely along sensory axons towards the nucleus in the neuronal cell body (8, 52). It was shown that HSV-1 capsids are cotransported to the neuronal nucleus with the tegument proteins VP1/2 (pUL36) and pUL37, which might support binding of capsids to dynein (8). Other tegument proteins including VP11/12, VP13/14, VP16, and VP22 were found predominantly lost prior to the onset of retrograde transport (8). However dissociation of HSV-1 VP16 and VP11/12 was not complete in all neurons, which is proposed to explain the establishment of an acute feedback loop in some neurons (8). After reaching the nuclear membrane, HSV-1 uncoating occurs at the nuclear pore complex (NPC), leading to the release of the viral genome through the NPC into the nuclear space (136). It is suggested that the viral DNA leaves the capsid through the

capsid portal, which is composed of pUL6 proteins (136). The portal proteins may also be implicated in mediating the proper orientation of the capsid and the NPC for release of the HSV-1 genome (136). The interaction of HSV-1 capsid with the NPC may occur via the inner tegument protein pUL25 (associated to the capsid) and one subunit of the NPC, CAN/Nup214 (136). In addition, the tegument protein pUL36, cellular Nup358 and importin beta, as well as the RanGTP/RanGDP cycle (for energy supply) seems to be involved in translocation of the viral genome into the nucleus (136). It is believed that the interaction with the NPC results in destabilization of the viral capsid, allowing the viral genome to leave the capsid due to pressure built up during the process of viral genome packaging (136). It is suggested that polarized DNA ejection into the nucleus occurs, in which one of the HSV-1 immediate early genes access the nucleus first (136). Transcription of this gene could act as a force pulling the genome out of the capsid (136). The internalized linear HSV-1 genome becomes circularized in the nucleus (59) and further serves as a template for both, viral gene expression and DNA replication (52).

### 3.3.4 HSV-1 transcription and translation

The production of HSV-1 proteins is regulated by temporal expression of the viral genes, subdivided into three different stages: immediate early (IE), early (E), and late (L; (171)). While IE and E genes encode for proteins required for the regulation of HSV-1 gene expression and DNA replication, L genes encode for proteins required for virus assembly and egress (170). L genes can be further divided into two classes, leaky-late and true late (7). The leaky-late genes are expressed at very low levels at early times after infection and become intensely upregulated at late times; in contrast, true late genes are expressed exclusively after the onset of viral DNA replication (7). The mechanism that restricts late gene activation after DNA replication has not yet been determined, although at least two general possibilities have been considered. First, the restriction may be related to DNA structure, or possibly DNA modifications that are altered immediately post replication. Alternatively, binding of viral or cellular proteins to repressor elements within late promoters may inhibit late gene expression, with subsequent displacement or titration of the repressors being accomplished by DNA replication.

All HSV-1 genes are transcribed by the host RNA polymerase II (52). Each viral promoter has a TATA box homology upstream of the start site of transcription (52). It was shown that initiation of HSV-1 gene expression requires demethylation of histones, which are associated with the viral DNA after entering the nucleus, by lysine-specific demethylase 1 (LSD1, (145). Further, VP16 together with LSD1 and the cellular factors Oct1 and HCFC1 mediate initial transcription of IE genes (145). At the same time, a repressor complex composed of LSD1, histone deacetylase (HDAC) 1 or 2, RE-1 silencing transcription factor (REST), and corepressor of REST (CoREST) prevent premature transcription of HSV-1 E and L genes (145). With the onset of IE gene transcription, ICP0 inactivates the repressor complex, allowing transcription of E and L genes (145). In addition, the cellular transcriptional activator Sp1 and the HSV-1 IE protein ICP4 are involved in activating transcription of E and L genes (90, 156), while at the same time ICP4 represses transcription from several IE

genes (17, 66, 103). ICP22, ICP27, and UL13 might also be implicated in transcriptional regulation, possibly via modification of RNA polymerase II activity (110). For example, ICP27 was found to directly interact with RNA polymerase II, which is suggested to prevent the expression of intron-containing cellular genes, while allowing the expression of intron-less viral genes (7). In this context, ICP27 has also a central function in shutting down the cellular mRNA splicing apparatus, via SRPK1 mediated inhibition of serine/arginine-rich splicing factors (SRSFs), which are required for splicesome assembly (153). To support viral mRNA stability and nuclear export, HSV-1 mRNAs become both 3'-polyadenylated and 5′-capped by host proteins (7). In addition, ICP27 was shown to be involved in polyadenylation and nucleocytoplasmic export of viral mRNAs (114).
HSV-1 protein synthesis is carried out by the host translation machinery (52). However, with some interference by HSV-1 proteins. For example, it was shown that binding of ICP0 to the elongation factor EF-1δ or hyperphosphorylation of EF-1δ by the HSV-1 protein kinase UL13 supports translation of viral mRNAs (88).

3.3.5 HSV-1 DNA replication and encapsidation.
Seven proteins encoded by HSV-1 were shown to be essential to promote viral DNA synthesis in cell culture: the origin-binding protein UL9, the ss DNA binding protein ICP8, the DNA polymerase (UL30/UL42), and the helicase/primase complex (UL5/UL8/UL52; (192)). The helicase-primase complex has DNA-dependent ATPase and helicase activity (29, 44, 92, 157) and the ability to synthesize primers (44, 157) in order to initiate viral replication. The HSV-1 ICP8 protein has a strong affinity to ss DNA (100) which supports an extended, single strand conformation of the HSV-1 DNA template (147) so that purine and pyrimidine bases of the template can base-pair readily with incoming nucleotides. In addition, ICP8 is involved in the recruitment and stimulation of replication factors and the expression of late virus genes (192), and ICP8 together with UL5 promote unwinding of the HSV-1 origin of DNA replication (33, 184, 186, 200). In addition to the minimal factors necessary for HSV-1 DNA replication, several accessory factors involved in nucleotide biosynthesis are supplied by the virus, including thymidine kinase, ribonucleotide reductase, deoxyuridine triphosphatase, and uracil-DNA-glycolsylase (192).
It was suggested that the formation of HSV-1 RCs starts adjacent to promyelocytic leukemia nuclear bodies (PML NBs; (111)). With the onset of HSV-1 gene expression PML NBs become disrupted (47). The disruption of these nuclear entities prevents their antiviral activity (47). At the same time, several PML proteins involved in transcriptional regulation or DNA replication and repair become associated with HSV-1 RCs including HDAC, Rb, p53, the MRN complex, ATM, ATR, RPA, Chk2, Rad52, and Brca1 (115–117, 171, 176, 194). In addition, a multitude of other DNA damage sensing and repair factors were found within HSV-1 RCs or associated with ICP8, especially proteins of the cellular homologous recombination machinery (115–117, 171, 176, 194, 196). The basic model for HSV-1 replication is a rolling-circle mechanism which leads to the formation of larger-than-unit length concatemers (19, 78, 79, 81). Additionally, complex, branched structures of HSV-1 replication

intermediates were observed (192). There is raising evidence that cellular recombination and repair proteins play a central role in multiple steps of HSV-1 DNA replication (105, 195). The function of several of these proteins is described in more detail below. Further support for efficient HSV-1 DNA replication may come from several chromatin remodeling factors (BRG1, BRM, hSNF2H, BAF155, mSin3a, and histone deacetylase 2) identified associated with HSV-1 RCs (171). These proteins may play a role in maintaining a nucleosome-free viral DNA, essential for virus replication (171). Although regulation of virus genes by cellular transcription factors seems to be reasonable, not all transcriptional regulators localized in HSV-1 RCs are involved in transcriptional regulation of virus genes. For example, the transcription factors Sp1 and Sp2 rather enhance OriS-dependent HSV-1 DNA replication than support virus transcription (1). In contrast, cellular transcriptional regulators Daxx and Sp100 are components of HSV-1 RCs and participate in the interferon mediated intrinsic antiviral response against HSV-1 (35, 48, 63). Recruitment of these factors into HSV-1 RCs may impede their antiviral activity via interferon response.
The products of HSV-1 DNA replication are larger-than-unit length concatemers (192). Both packaging signals *pac1* and *pac2* lie within the *a* repeats of the HSV-1 genome (Fig. 2B; (52)). The tripartite terminase as well as UL32 and UL28 recognize the genomic ends in concatemers and endonucleolytically cleave these into precise genomic lengths, adjacent to the *pac* signals in the DR region of the genome (11, 52). One single genome is then inserted into the capsid through the portal, supported by the ATPase-activity of the terminase (11). The inserted DNA replaces the inner scaffold proteins, which are degraded or expelled during DNA packaging (11).

### 3.3.6 HSV-1 egress.

There are different hypotheses concerning the mechanism of HSV-1 egress. A widespread view is that the newly synthesized capsids, together with some tegument proteins bud into the perinuclear lumen, mediated by a virus encoded budding machinery (including UL34 and UL31) located at the inner nuclear membrane (11, 52). In a second step, the immature primary enveloped virions fuse with the outer nuclear membrane and release the capsid into the cytoplasm. The capsid then buds into membranes of the late trans-Golgi compartment or endosome, thereby acquiring the final envelope (52). An alternative pathway describes the nuclear exit of naked capsids via impaired nuclear pores (134). In a third alternative mechanism, after budding into the perinuclear lumen (from the cytoplasm), the virion follows the intraluminal transport via the endoplasmatic reticulum (ER) and the Golgi apparatus (134). Regardless of the mechanism of envelopment, virions exit the cell by exocytosis (52, 134). In case of HSV-1 replication within neurons, the nucleocapsid and envelop proteins are transported separately to axon termini and secondary envelopment is suggested to occur at the site of virion egress (52).

### 3.3.7 HSV-1 latency.

Virus replication in epithelial cells and virion release at the site of host entry can lead to the spread of HSV-1 into neurons of the trigeminal ganglia via axon termini. Upon entry, virions can travel via retrograde axonal transport to the cell bodies where

lifelong latency is established. The mechanisms that control productive or latent infection are not fully understood, however a hallmark of HSV-1 latent infection is that transcription of latency-associated transcripts (LATs) are the only viral products synthesized in large quantities from latent HSV-1 episomes (52). A primary LAT transcript of ~8.5kb is processed into a ~2kB intron (49) and several microRNAs (175). While the function of the LAT intron is not clear, several of the microRNAs are involved in repression of viral mediators of lytic infection, including ICP0 and ICP4 (175). The structure of the LAT intron closely resembles cellular long non-coding RNAs (lncRNAs), and it may therefore be involved in epigenetic regulation, similar to the function of cellular lncRNAs (198). In the host, reactivation of the virus can be induced by unknown causes. A recent study showed that the treatment of axon termini of latently infected neurons with the mTOR kinase inhibitor rapamycin stimulated lytic replication of HSV-1 (93). Since mTOR signaling pathways in neurons have been shown to integrate a variety of extracellular signals into neurons (193), these results indicate that environmental stimuli may be sufficient to induce reactivation of HSV-1 from latency. In line with this concept, mTOR activity was shown to regulate the translational repressor eIF4E-binding protein (4E-BP), which is involved in the regulation of expression of latent HSV-1 genomes (93).

## 3.4 Viruses and the cellular DNA damage sensing and repair machinery.

There is rising evidence that viruses which replicate in the host cell nucleus interfere with nuclear components and pathways, in order to facilitate their own propagation (82, 107, 130, 174, 187). DNA-damage response (DDR) pathways in particular may play central roles in virus infection (82, 107, 130, 174, 187). The host cell responds to incoming viral genomes during the first few hours of infection, by activating a DNA damage response (DDR; (187, 189)). This response is facilitated by the presence of free ss DNA and/or ds DNA ends as well as unusual secondary structures of viral genomes (e.g. the hairpins of the AAV2 ITRs, see above and ref. (52)) and/or extraordinary replication intermediates (e.g. branched structures of the HSV-1 genome, see above and ref. (192)). In addition, similar to replication of the host cell DNA, replication of virus DNA, once initiated, may not proceed to completion without interruptions at both leading- and lagging-strand synthesis (34), resulting in the initiation of DDR signaling. Several studies have demonstrated that these cellular proteins of the DNA damage sensing and repair machinery can have positive and/or negative effects on the viral life cycle, depending on the virus and its replication strategy (82, 107, 130, 174, 187). For instance, DDR-mediated covalent linkage of Ad DNA would inhibit packaging; therefore, Ad inactivates specific DDR proteins by degradation or mislocalization (DNA-PK, DNA ligase IV, and the Mre11 complex; (12, 27, 152)). In contrast, HSV-1 uses the DNA damage sensing and repair machinery to promote covalent linkage of its genome during replication (discussed in more detail below and ref. (192)). In addition, the DDR pathway can be a tool for the viruses in general, to gain control over the cell cycle and to allow viral genome replication, while preventing premature apoptosis of infected cells (82, 107, 130, 174, 187). Furthermore, DNA-damage induced protein complexes may protect viral DNA from nuclease-dependent degradation (82, 107, 130, 174, 187). Therefore, viruses have evolved strategies to manipulate the DNA damage sensing and repair machinery in a way that detrimental factors become unplugged while beneficial factors become hijacked, leading to a dramatic remodeling process in the host nucleus which is described below.

### 3.4.1 The main players of the cellular DNA damage sensing and repair machinery.

Cells activate a complex signaling network that includes kinase regulation, transcriptional induction and redistribution of a multitude of factors in response to DNA damage or stalled replication forks (61). Depending on the extent of DNA damage, cell cycle progression is stopped (99), to enable DNA repair, or apoptosis is induced via induction and activation of Bax (65, 143). The three main kinases, mediating signaling transmission after recognizing DNA damage or stalled replication forks are PI3 kinase-like kinases (PIKKs), specifically ataxia telangiectasia mutated (ATM), ATM and Rad3-related kinase (ATR), and DNA-dependent protein kinase (DNA-PK; (166)). These three kinases are recruited to sites of DNA damage and play a crucial role in DDR by transmitting and amplifying the signal (166).

3.4.2 The ATR pathway and stalled replication forks.

DNA damage pathways contribute to proper replication of cellular DNA by stabilizing transiently stalled replication forks, regulating replication after DNA repair restart, or facilitating fork movement on difficult-to-replicate templates (83). At replication forks, progression of the DNA polymerases is uncoupled from the helicase activity, leading to the formation of ss DNA gaps, which become immediately coated by the cellular ss DNA-binding protein replication protein A (RPA) and which are further recognized by the ATR-ATRIP (ATR interacting protein) complex (53, 83). The subsequently induced DDR is crucial to modulate DNA replication dynamics in order to prevent DNA damage during replication (83). ATR signaling via checkpoint kinase 1 (Chk1) leads to the activation and recruitment of the repair machinery as well as checkpoint activation, mediating a cell cycle arrest (53, 83). Therefore, Chk1 acts as a signaling transmitter to signal DNA damage to the rest of the nucleus. The adaptor protein claspin is crucial for bringing ATR and Chk1 together (39). Additional proteins implicated in the conserved ATR checkpoint pathway are: the RAD17–RFC complex (RAD17, RFC2, RFC3, RFC4, RFC5), the 9-1-1 complex (RAD9, RAD1, HUS1) and the ATR activator protein TopBP1 (53). However, the mechanism by which TOPBP1 activates ATR is poorly defined. If DNA repair is not necessary, the MCM helicase complex, the WRN (Werner syndrome, RecQ helicase-like), and BLM helicases are able to remodel perturbed replication fork structures or difficult-to-replicate secondary structures to aid progression of the polymerase complex in response to ATR signaling (83).

Besides replication stress, ATR is also activated in response to many types of DNA damage, including DSBs, base adducts, or crosslinks, however a single DNA structure might be always responsible for activation of ATR kinase activity (39).

3.4.3 ATM and DNA-PK in homologous recombination (HR) and non-homologous end joining (NHEJ) after recognition of DNA double strand breaks.

In contrast to ATR, DNA-PK is not able to arrest the replication process following replication stress (108) but plays an essential role in DNA ds break repair (159). DNA ds breaks can arise from endogenous sources, including reactive oxygen species (ROS), cellular nucleases, and collapsed replication forks, but also from exogenous sources such as ionizing radiation and chemicals (159). Two main pathways are involved in repairing DNA ds breaks: homologous recombination (HR) and non-homologous end joining (159). Several DNA repair factors are common to both pathways, including the MRN (Mre11/Rad50/Nbs1) complex, BRCA1, H2AX, PARP-1, and RAD18 (159). The MRN complex acts as main sensor of ds breaks (99) to induce not only HR or NHEJ, but also for example cell-cycle arrest (99). Cellular DNA damage repair via HR can only occur in S or G2 phase of the cell cycle when sister chromatids are available (159). NHEJ remains active throughout the cell cycle, indicating that NHEJ competes for ds breaks in S and G2 phase of the cell cycle (159). The core machinery of HR is built by CtIP, RPA, EXO1, BRCA 1 and 2, PALB2, Rad51, Rad52, and Rad54 , while the core repair machinery of NHEJ is composed of DNA-PK (Ku70/Ku80/DNA-PKcs), Artemis, Lig4, XRCC4, 53BP1, and

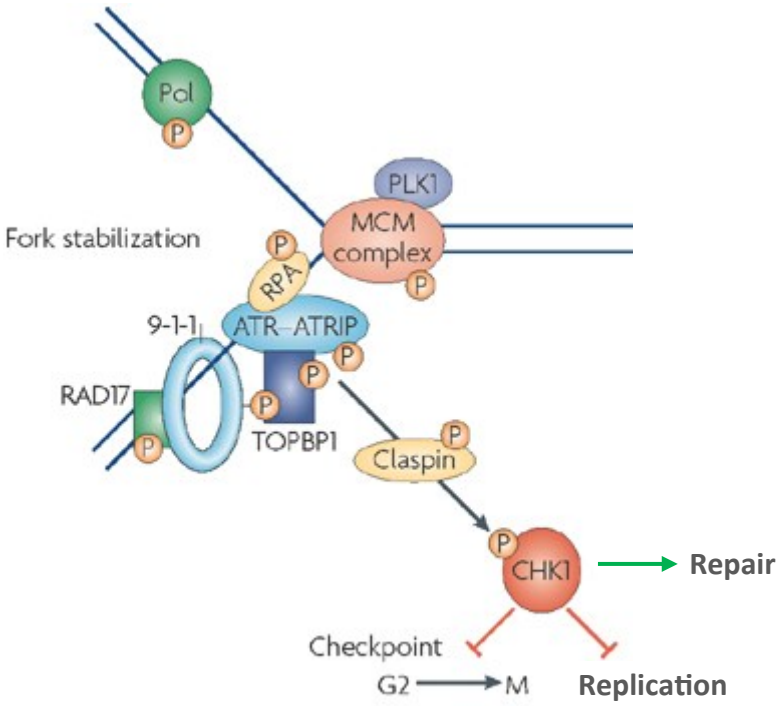



**Fig 3.** ATR mediated DDR signaling at stalled replication forks. At stalled replication forks ss DNA gaps become immediately coated with RPA and are further recognized by the ATR-ATRIP complex. Recruitment of TOPBP1 by the 9-1-1 complex leads to activation of ATR kinase activity. The adaptor protein claspin then supports ATR mediated acntivation of Chk1 which transmitts the DNA damage signal to the rest of the nucleus. Finally this ATR mediated signaling at a DNA lesions or stalled replication forks can coordinate cell cycle, repair, and replication. Figure adapted from: Nature Reviews Molecular Cell Biology 9, 616-627 (August 2008) | doi:10.1038/nrm2450.

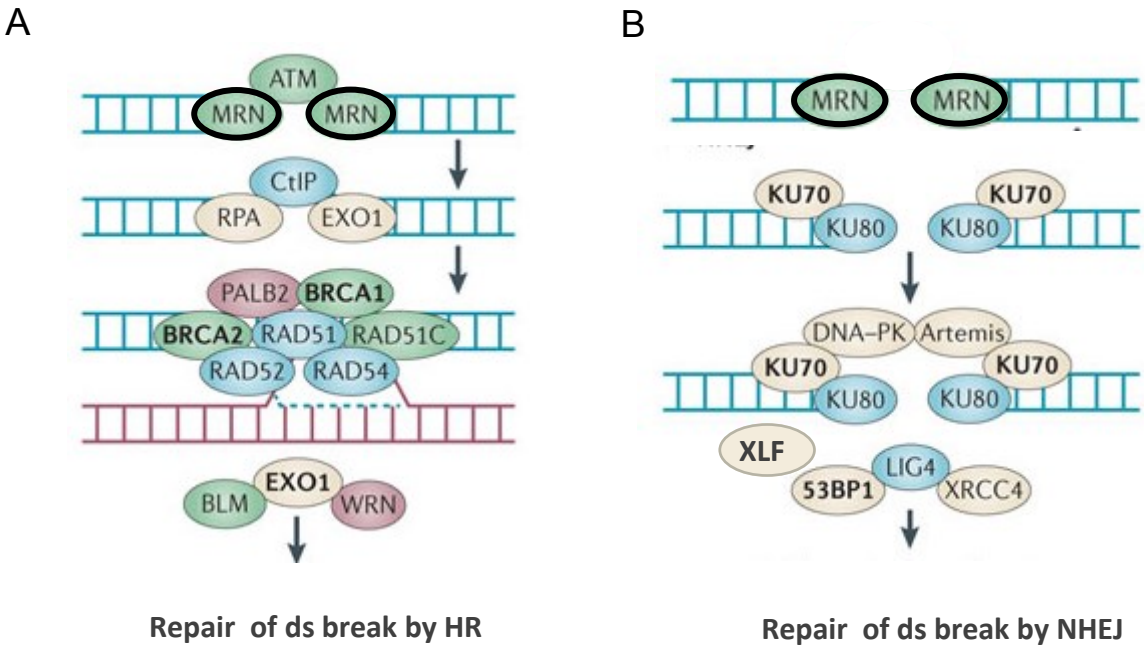



**Fig 4.** ATM and DNA-PK in HR (A) and NHEJ (B). The MRN complex plays an important role in sensing DNA ds breaks in both HR and NHEJ. ATM is further responsible to recruit CtIP to sites of DNA damage. The core repair machinery of HR is finally composed of CtIP, RPA, EXO1, BRCA 1 and 2, PALB2, Rad51, Rad52, and Rad54 (A), while the core repair machinery of NHEJ is composed of DNA-PK (Ku70/Ku80/DNA-PKcs), Artemis, Lig4, XRCC4, 53BP1 and XLF. Figure adapted from: Nature Reviews Cancer 12, 587-598 (September 2012) | doi:10.1038/nrc3342.

XLF (26, 34). In HR, WRN and BLM were found to stimulate endonuclease activity of EXO1 at ssDNA–dsDNA junction as well as the incision of a nucleotide at a nick (154) and 5′→3′ ds DNA exonuclease activity (131), respectively. ATM plays not only a central role in HR by recruiting CtIP to sites of DNA damage (34), several proteins involved in initial DDR signaling are targets for phosphorylation of both ATM and DNA-PKcs. For example, activation of Chk2 by ATM after ds breaks is well described (4, 37, 87), but also DNA-PK was reported to induce activation of Chk2 (104, 173). In addition, p53 was shown to be a target of both DNA-PK (102, 180, 183) and ATM (14). Due to this overlap it seems to be likely that initial DNA-PK mediated signaling influences HR and that ATM signaling contributes to NHEJ. However, it is important to mention that besides ATM and DNA-PK, ATR signaling can also become activated in response to the formation of ss DNA gaps on DNA double strand ends or repair intermediates (53) and may therefore also contribute to HR or NHEJ events.

3.4.4 HSV-1 and the cellular DNA damage sensing and repair machinery.

HSV-1 induces the activation of a cellular DNA ds break response pathway including ATM, the MRN complex, Chk2, p53, RPA, and Rad51 (25, 105, 158, 196). It is suggested that the MRN complex in particular is required for the formation and stability of the concatemers and complex branched structures observed during HSV-1 genome replication (105). Several proteins found in HSV-1 RCs belong to the cellular HR machinery. It is suggested that recombination events play a central role in HSV-1 replication, by promoting inversion of two segments of the HSV-1 genome upon replication and the formation of HSV-1 concatemers (195). However, not all components of the HR machinery have a significant role in HSV-1 replication. The WRN complex was shown to have only a modest influence on HSV-1 recombination events (171). In contrast to several HR proteins which might support HSV-1 replication, proteins of the NHEJ seem to have inhibitory function in HSV-1 replication (135, 171). Although Ku70 was found as component of HSV-1 RCs, HSV-1 replication in absence of Ku70 was shown to be significantly increased (171). It is unclear whether any specific HSV-1 proteins counteract Ku70 activity. In addition, another NHEJ protein, DNA-PKcs, was shown to reduce replication efficiency of HSV-1 in cell culture experiments (135). Therefore, it is not surprising that HSV-1 targets DNA-PKcs for degradation, using the E3 ubiquitin ligase function of the virus protein ICP0 (101, 135). It was suggested that the NHEJ pathway in general may have an inhibitory function on HSV-1 replication, for example by blocking homologous recombination events of the ds DNA genome of HSV-1 (171). However more recent results showed that knockdown of another central element of NHEJ, DNA ligaseIV/XRCC4, reduced HSV-1 replication severely (120). It is proposed that DNA ligase IV/XRCC4 contributes to the initial formation of end-to-end ligation of the incoming viral genomes, forming circular viral molecules for replication (120). All together, these data indicate that the NHEJ machinery may be useful for HSV-1 replication, however, in a strictly time dependent manner.

While ATM and ATM-targets were observed to be activated during HSV-1 infection, ATR and ATR-targets were not. This has been proposed to be a result of disruption

of the ATR/ATRIP protein complex by the HSV-1 IE protein ICP0 (197). However, later studies showed that although not active, both ATR and ATRIP were recruited into HSV-1 RCs (116, 176). It was also shown that proteins of the ATR pathway (in absence of ATR kinase activity) including ATR, RPA, TopBP1, clapsin, or CINP play a beneficial role in HSV-1 gene expression and virus production (115). By supporting gene expression rather than mediating a DDR, it seems that HSV-1 uses cellular proteins of the ATR pathway outside of their host context.
Although an ATR-mediated DDR was not observed during HSV-1 infection, several proteins related to stalled replication forks were found associated to HSV-1 RCs, including WRN, Rad51, BRCA1 Rad17, Rad9, or HUS1 (115, 196). The appearance of stalled replication forks during HSV-1 genome replication is very likely, due to described nicks and gaps (57, 89). Similar to their cellular function (83), these proteins might support also stabilization of the viral replication forks and restart of interrupted replication. Therefore, in absence of these cellular factors, the integrity of viral replication forks or replication intermediates may not be ensured. It is proposed that such a mechanism is involved also in the establishment of HSV-1 latency in neurons (105). Indeed, it was shown that in neurons, HSV-1 failed to initiate both a DDR and the formation of viral replication centers (105). However, although viral infection of neuroblastoma cells did not induce a DDR, treatment of these cells with cellular DNA damage inducers facilitated HSV-1 promoter activity and viral protein expression (177). This observation also implicates a mechanism by which HSV-1 genes, in a quiescent or latent state, may become active to facilitate productive infection through activation of the cellular DNA damage sensing and repair machinery in neuronal cells (177).

3.4.5 AAV2 and the cellular DNA damage sensing and repair machinery.
In addition to helper virus proteins, the fate of AAV2 replication depends also on cellular proteins. Recently, cellular proteins have been identified that interact with AAV2 Rep 78/68 during Ad or HSV-1 supported AAV2 replication (123, 128). With both helper viruses, one of the main groups of cellular proteins belongs to the cellular DNA damage sensing and repair machinery (123, 128). The function of these proteins in AAV2 replication is reviewed in detail below (3.6).

3.4.6 DDR induced by AAV2 in absence of a helper virus.
Early studies have shown that infection with wtAAV2 or UV light-inactivated AAV2 (UV-AAV2) inhibits multiplication of the cells in culture and that the perturbation of the cell cycle leads to the accumulation of cells in late S and/or G2 phases, independent on viral gene expression and replication (199). It is likely that the ss AAV2 DNA and its hairpin shaped ITRs are the primary signals for a virus-induced cell cycle arrest. Further examination revealed that AAV2 DNA triggers a DDR that resembles the response of an aberrant cellular DNA replication fork (85). The signaling was shown to be strictly dependent on ATR and Chk1 functions in presence of BML, Rad9, and TopBP1 (85). Moreover, DNA polymerase delta, RPA, and the Rad9/Rad1/Hus1 complex but not ATM and NBS1 have been found accumulated in AAV2 DNA foci (85). The hypothetical model of Jurvansuu et al. suggests primary recognition of

AAV2 DNA by the ss DNA binding proteins Rad51 and RPA (85). RPA further enables binding of both ATR/ATRIP and 9-1-1 protein complexes to AAV2 DNA (85). ATR phosphorylates Rad17, 9-1-1 and Chk1 and recruits other proteins, which leads to the induction of a cell cycle arrest (85). Additional studies revealed that p53 plays also an essential role in the induction of the AAV2 induced cell cycle arrest, as cells deficient in p53 were shown to proceed into lethal mitosis, independent of the presence or absence of AAV2 Rep proteins (84, 142). By inducing cell death in cells defective for p53, AAV2 was shown to inhibit tumor growth in mice (142). Therefore AAV2 is suggested a very promising tool to deliver DNA of unusual structure into cells in order to trigger a DNA damage response that selectively eliminates tumor cells lacking p53 activity (142).

Despite the AAV2 DNA, the AAV2 replication proteins Rep 78 and Rep 68, but not Rep 52 and Rep 40, have been shown to induce a cell cycle arrest in G1 and G2 phase of the cell cycle, mediated by elevated levels of the CDK inhibitor p21 and reduced levels of cyclin E-, A- and B1-associated kinase activity (150). Expression of Rep 78 has been shown to interfere also with S-phase progression, mediated by accumulation of hypophosphorylated retinoblastoma protein (Rb; (150)). The antiproliferative effect of Rep 78/68 on cells was also linked to the inhibition of Cdc25A (22). In addition, a potential Rep 78/68-induced damage of the cellular chromatin is suggested to activate a cellular DDR (22); however further studies supporting this hypothesis are necessary.

### 3.4.7 The role of DNA damage sensing and repair proteins in AAV2 vector transduction.

The DNA sensing and repair machinery is not only affecting wtAAV2 replication, but also important steps in rAAV2 vector transduction, including conversion of the ss AAV2 vector genome into ds DNA, formation of stable ds circular monomers or large concatemers (circular and/or linear), and efficient transgene expression in target cells (2). Inhibitory effects of the MRN complex (32, 152) and ATM (38, 149) on rAAV2 transduction were also observed. The inhibitory effect of ATM was shown to correlate with reduced circularization of rAAV2 genomes (149). Another study showed that ATM together with Ku86 is linked to rAAV2 processing, resulting in reduced amounts of stable episomal rAAV2 vectors and, therefore, inefficient long-term rAAV2 vector gene expression (38). However, when using a self-complementary recombinant AAV2 (scAAV2) vector, the MRN complex and ATM together with RecQ and DNA-PK were shown to support efficient transduction (38), indicating that the inhibitory effect of ATM and Ku86 affects second-strand synthesis or the stabilization of ss AAV2 vectors.

The inhibitory effect of DDR factors on AAV2 vector transduction might also include several other steps of AAV2 infection and is suggested to belong to the cell's intrinsic antiviral response (46, 47, 106, 169). In this regard, one of the functions of the helper virus might be to overcome these cellular barriers. One example for this hypothesis is the Ad mediated degradation of the MRN complex discussed below (152).

*Of note: A chapter on viral and cellular components of AAV2 replication compartments completes the introduction of this thesis. However, as this topic is presented here in the form of a manuscript that includes its own references, it has been placed after chapter 3.5, which lists the references for chapters 3.1-3.4.*

3.5 References (chapters 3.1-3.4)


1. **A T Nguyen-Huynh, and P. A. Schaffer.** 1998. Cellular transcription factors enhance herpes simplex virus type 1 oriS-dependent DNA replication. J Virol **72**:3635–3645.
2. **Adachi, K., and H. Nakai** (ed.). 2011. The Role of DNA Repair Pathways in Adeno-Associated Virus Infection and Viral Genome Replication / Recombination / Integration. DNA Repair and Human Health. InTech, New York, NY.
3. **Aghi, M., and R. L. Martuza.** 2005. Oncolytic viral therapies – the clinical experience. Oncogene **24**:7802–7816.
4. **Ahn, J. Y., J. K. Schwarz, H. Piwnica-Worms, and C. E. Canman.** 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res **60**:5934–5936.
5. **Akhtar, J., and D. Shukla.** 2009. Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry. FEBS J **276**:7228–7236.
6. **Alazard-Dany, N., A. Nicolas, A. Ploquin, R. Strasser, A. Greco, A. L. Epstein, C. Fraefel, A. Salvetti, and P. O'Hare.** 2009. Definition of Herpes Simplex Virus Type 1 Helper Activities for Adeno-Associated Virus Early Replication Events. PLoS Pathog **5**:e1000340.
7. **Anders, D., J. Kerry, and G. Pari** (ed.). 2007. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Chapter 19. DNA synthesis and late viral gene expression. Cambridge University Press, Cambridge, New York.
8. **Antinone, S. E., and G. A. Smith.** 2010. Retrograde Axon Transport of Herpes Simplex Virus and Pseudorabies Virus: a Live-Cell Comparative Analysis. J Virol **84**:1504–1512.
9. **Asokan, A., J. B. Hamra, L. Govindasamy, M. Agbandje-McKenna, and R. J. Samulski.** 2006. Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry. J Virol **80**:8961–8969.
10. **Atchison RW, Casto BC, and Hammon WM.** 1965. Adenovirus-Associated Defective Virus Particles Adenovirus-Associated Defective Virus Particles. Science **149**:754–756.
11. **Baines, J. D.** 2011. Herpes simplex virus capsid assembly and DNA packaging: a present and future antiviral drug target. Trends in Microbiology **19**:606–613.
12. **Baker, A., K. J. Rohleder, L. A. Hanakahi, and G. Ketner.** 2007. Adenovirus E4 34k and E1b 55k Oncoproteins Target Host DNA Ligase IV for Proteasomal Degradation. J Virol **81**:7034–7040.
13. **Baltimore, D.** 1971. Expression of animal virus genomes. Bacteriol Rev **35**:235–241.
14. **Banin, S., L. Moyal, S. Shieh, Y. Taya, C. W. Anderson, L. Chessa, N. I. Smorodinsky, C. Prives, Y. Reiss, Y. Shiloh, and Y. Ziv.** 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science (New York, N.Y **281**:1674–1677.
15. **Bartlett, J. S., R. Wilcher, and R. J. Samulski.** 2000. Infectious Entry Pathway of Adeno-Associated Virus and Adeno-Associated Virus Vectors. J Virol **74**:2777–2785.
16. **Batchu, R. B., M. A. Shammas, J. Y. Wang, R. J. Shmookler Reis, and N. C. Munshi.** 2002. Interaction of adeno-associated virus Rep78 with SV40 T antigen: implications in Rep protein expression leading to the inhibition of SV40-mediated cell proliferation. Intervirology **45**:115–118.

17. **Beard, P., S. Faber, K. Wilcox, and L. Pizer.** 1983. Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. Proc Natl Acad Sci U S A **11**:4016–4020.
18. **Becerra, S. P., J. A. Rose, M. Hardy, B. M. Baroudy, and C. W. Anderson.** 1985. Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon. Proc. Natl. Acad. Sci. U.S.A. **82**:7919–7923.
19. **Ben-Porat, T., and S. A. Tokazewski.** 1977. Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. Virology **79**:292–301.
20. **Berns, K. I., and R. A. Bohenzky.** 1987. Adeno-associated viruses. an update. Advances in virus research **32**:243–306.
21. **Berns, K. I., and R. M. Linden.** 1995. The cryptic life style of adeno-associated virus. Bioessays **17**:237–245.
22. **Berthet, C., K. Raj, P. Saudan, and P. Beard.** 2005. How adeno-associated virus Rep78 protein arrests cells completely in S phase. Proceedings of the National Academy of Sciences of the United States of America **102**:13634–13639.
23. **Bjorklund, A., D. Kirik, C. Rosenblad, B. Georgievska, C. Lundberg, and R. J. Mandel.** 2000. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res **886**:82–98.
24. **Bohenzky, R. A., R. B. LeFebvre, and K. I. Berns.** 1988. Sequence and symmetry requirements within the internal palindromic sequences of the adeno-associated virus terminal repeat. Virology **166**:316–327.
25. **Boutell, C., and R. D. Everett.** 2004. Herpes simplex virus type 1 infection induces the stabilization of p53 in a USP7- and ATM-independent manner. J Virol **78**:8068–8077.
26. **Bouwman, P., and J. Jonkers.** 2012. The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. Nat Rev Cancer **12**:587–598.
27. **Boyer, J., K. Rohleder, and G. Ketner.** 1999. Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. Virology **263**:307–312.
28. **Brister, J. R., and N. Muzyczka.** 2000. Mechanism of Rep-mediated adeno-associated virus origin nicking. J Virol **74**:7762–7771.
29. **Calder, J. M., and N. D. Stow.** 1990. Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. Nucleic Acids Res **18**:3573–3578.
30. **Cao, M., H. Zhu, S. Bandyopadhyay, H. You, and P. L. Hermonat.** 2011. HPV-16 E1, E2 and E6 each complement the Ad5 helper gene set, increasing rAAV2 and wt AAV2 production. Gene Ther **19**:418–424.
31. **Cataldi, M. P., and D. M. McCarty.** 2010. Differential effects of DNA double-strand break repair pathways on single-strand and self-complementary adeno-associated virus vector genomes. J Virol **84**:8673–8682.
32. **Cervelli, T., J. A. Palacios, L. Zentilin, M. Mano, R. A. Schwartz, M. D. Weitzman, and M. Giacca.** 2008. Processing of recombinant AAV genomes occurs in specific nuclear structures that overlap with foci of DNA-damage-response proteins. Journal of cell science **121**:349–357.
33. **Challberg, M. D.** 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc Natl Acad Sci U S A **83**:9094–9098.

34. **Chapman, J. R., M. R. Taylor, and S. J. Boulton.** 2012. Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. Molecular Cell **47**:497–510.
35. **Chee, A. V., P. Lopez, P. P. Pandolfi, and B. Roizman.** 2003. Promyelocytic Leukemia Protein Mediates Interferon-Based Anti-Herpes Simplex Virus 1 Effects. J Virol **77**:7101–7105.
36. **Chejanovsky, N., and B. J. Carter.** 1989. Mutagenesis of an AUG codon in the adeno-associated virus rep gene: effects on viral DNA replication. Virology **173**:120–128.
37. **Chen, Y., and Y. Sanchez.** 2004. Chk1 in the DNA damage response. conserved roles from yeasts to mammals. DNA repair **3**:1025–1032.
38. **Choi, V. W., D. M. McCarty, and R. J. Samulski.** 2006. Host Cell DNA Repair Pathways in Adeno-Associated Viral Genome Processing. J Virol **80**:10346–10356.
39. **Cimprich, K. A., and D. Cortez.** 2008. ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol **9**:616–627.
40. **Clement, C., V. Tiwari, P. M. Scanlan, T. Valyi-Nagy, B. Y. J. T. Yue, and D. Shukla.** 2006. A novel role for phagocytosis-like uptake in herpes simplex virus entry. J Cell Biol **174**:1009–1021.
41. **Costello, E., P. Saudan, E. Winocour, L. Pizer, and P. Beard.** 1997. High mobility group chromosomal protein 1 binds to the adeno-associated virus replication protein (Rep) and promotes Rep-mediated site-specific cleavage of DNA, ATPase activity and transcriptional repression. EMBO J **16**:5943–5954.
42. **Cotmore, S. F., and P. Tattersall.** 1996. 28 Parvovirus DNA Replication. Cold Spring Harbor Monograph Archive; Volume 31 (1996): DNA Replication in Eukaryotic Cells.
43. **Daya, S., N. Cortez, and K. I. Berns.** 2009. Adeno-associated virus site-specific integration is mediated by proteins of the nonhomologous end-joining pathway. J Virol **83**:11655–11664.
44. **Dodson, M. S., and I. R. Lehman.** 1991. Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. Proc Natl Acad Sci U S A **88**:1105–1109.
45. **Duan, D., Z. Yan, Y. Yue, W. Ding, and J. F. Engelhardt.** 2001. Enhancement of muscle gene delivery with pseudotyped adeno-associated virus type 5 correlates with myoblast differentiation. J Virol **75**:7662–7671.
46. **Everett, R. D., J. Murray, A. Orr, and C. M. Preston.** 2007. Herpes simplex virus type 1 genomes are associated with ND10 nuclear substructures in quiescently infected human fibroblasts. J Virol **81**:10991–11004.
47. **Everett, R. D., S. Rechter, P. Papior, N. Tavalai, T. Stamminger, and A. Orr.** 2006. PML Contributes to a Cellular Mechanism of Repression of Herpes Simplex Virus Type 1 Infection That Is Inactivated by ICP0. J Virol **80**:7995–8005.
48. **Everett, R. D., D. F. Young, R. E. Randall, and A. Orr.** 2008. STAT-1- and IRF-3-Dependent Pathways Are Not Essential for Repression of ICP0-Null Mutant Herpes Simplex Virus Type 1 in Human Fibroblasts. J Virol **82**:8871–8881.
49. **Farrell, M. J., A. T. Dobson, and L. T. Feldman.** 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc Natl Acad Sci U S A **88**:790–794.
50. **Ferrari, F. K., T. Samulski, T. Shenk, and R. J. Samulski.** 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J Virol **70**:3227–3234.

51. **Fisher, K. J., G. P. Gao, M. D. Weitzman, R. DeMatteo, J. F. Burda, and J. M. Wilson.** 1996. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. J Virol **70**:520–532.
52. **Flint, S. J.** (ed.). 2004. Principles of virology. Molecular biology, pathogenesis, and control of animal viruses, 2nd ed. ASM Press, Washington, D.C.
53. **Flynn, R. L., and L. Zou.** 2011. ATR: a master conductor of cellular responses to DNA replication stress. Trends in Biochemical Sciences **36**:133–140.
54. **Forterre, P.** 2006. The origin of viruses and their possible roles in major evolutionary transitions. Virus Res. **117**:5–16.
55. **Fraefel, C., A. G. Bittermann, H. Bueler, I. Heid, T. Bachi, and M. Ackermann.** 2004. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. J Virol **78**:389–398.
56. **Francois, A., M. Guilbaud, R. Awedikian, G. Chadeuf, P. Moullier, and A. Salvetti.** 2005. The cellular TATA binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 p5 element. J Virol **79**:11082–11094.
57. **Frenkel, N., and B. Roizman.** 1971. Herpes vimplex virus: genome size and redundancy studied by renaturation kinetics. J Virol **8**:591–593.
58. **Gao, G., L. Vandenberghe, and Wilson J.** 2005. New recombinant serotypes of AAV2 vectors. Gene Ther **5**:285–297.
59. **Garber, D. A., S. M. Beverley, and D. M. Coen.** 1993. Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. Virology **197**:459–462.
60. **Gianni, T., G. Campadelli-Fiume, and L. Menotti.** 2004. Entry of Herpes Simplex Virus Mediated by Chimeric Forms of Nectin1 Retargeted to Endosomes or to Lipid Rafts Occurs through Acidic Endosomes. J Virol **78**:12268–12276.
61. **Giglia-Mari, G., A. Zotter, and W. Vermeulen.** 2011. DNA damage response. Cold Spring Harbor perspectives in biology **3**:a000745.
62. **Girod, A., C. E. Wobus, Z. Zadori, M. Ried, K. Leike, P. Tijssen, J. A. Kleinschmidt, and M. Hallek.** 2002. The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. J Gen Virol **83**:973–978.
63. **Glass, M., and R. D. Everett.** 2013. Components of Promyelocytic Leukemia Nuclear Bodies (ND10) Act Cooperatively To Repress Herpesvirus Infection. J Virol **87**:2174–2185.
64. **Glauser, D. L., O. Saydam, N. A. Balsiger, I. Heid, R. M. Linden, M. Ackermann, and C. Fraefel.** 2005. Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. J Virol **79**:12218–12230.
65. **Gong, B., Q. Chen, B. Endlich, S. Mazumder, and A. Almasan.** 1999. Ionizing radiation-induced, Bax-mediated cell death is dependent on activation of cysteine and serine proteases. Cell Growth Differ **10**:491–502.
66. **Gu, B., R. Kuddus, and N. A. DeLuca.** 1995. Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. Mol Cell Biol **15**:3618–3626.
67. **H Lechevalier.** 1972. Dmitri Iosifovich Ivanovski (1864-1920). Bacteriol Rev. **36**:135–145.
68. **Hamilton, H., J. Gomos, K. I. Berns, and E. Falck-Pedersen.** 2004. Adeno-Associated Virus Site-Specific Integration and AAVS1 Disruption. J Virol **78**:7874–7882.

69. **Hefferon, K. L.** 2006. Recent advances in DNA virus replication 2006. Research Signpost, Trivandrum, Kerala, India.
70. **Heilbronn, R., M. Engstler, S. Weger, A. Krahn, C. Schetter, and M. Boshart.** 2003. ssDNA-dependent colocalization of adeno-associated virus Rep and herpes simplex virus ICP8 in nuclear replication domains. Nucleic acids research **31**:6206–6213.
71. **Hermonat, P. L. e. a.** 1998. The adeno-associated virus Rep78 major regulatory protein binds the cellular TATA-binding protein in vitro and in vivo. Virology.:120–127.
72. **Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka.** 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J Virol **51**:329–339.
73. **Holscher, C., J. A. Kleinschmidt, and A. Burkle.** 1995. Holscher, C 1995High-level expression of adeno-associated virus (AAV) Rep78 or Rep68 protein is sufficient for infectious-particle formation by a rep-negative AAV mutant. J Virol **69**:6880–6885.
74. **Homa, and Brown.** 1997. Capsid assembly and DNA packaging in herpes simplex virus. Rev Med Virol **7**:107–122.
75. **Hunter, L. A., and R. J. Samulski.** 1992. Colocalization of adeno-associated virus Rep and capsid proteins in the nuclei of infected cells. J Virol **66**:317–324.
76. **Hüser, D., A. Gogol-Döring, T. Lutter, S. Weger, K. Winter, E.-M. Hammer, T. Cathomen, K. Reinert, R. Heilbronn, and B. R. Cullen.** 2010. Integration Preferences of Wildtype AAV-2 for Consensus Rep-Binding Sites at Numerous Loci in the Human Genome. PLoS Pathog **6**:e1000985.
77. **International Committee on Taxonomy of Viruses., and A. M. King.** 2012. Virus taxonomy : classification and nomenclature of viruses : ninth report of the International Committee on Taxonomy of Viruses. Academic Press, London.
78. **Jacob, R. J., L. S. Morse, and B. Roizman.** 1979. Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J Virol **29**:448–457.
79. **Jacob, R. J., and B. Roizman.** 1977. Anatomy of herpes simplex virus DNA VIII. Properties of the replicating DNA. J Virol **23**:394–411.
80. **Janik, J. E., M. M. Huston, and J. A. Rose.** 1981. Locations of adenovirus genes required for the replication of adenovirus-associated virus. Proc. Natl. Acad. Sci. U.S.A. **78**:1925–1929.
81. **Jean, J. H., M. L. Blankenship, and T. Ben-Porat.** 1977. Replication of herpesvirus DNA. I. Electron microscopic analysis of replicative structures. Virology **79**:281–291.
82. **Jiang, M., and M. J. Imperiale.** 2012. Design stars: how small DNA viruses remodel the host nucleus. Future Virology **7**:445–459.
83. **Jones, R. M., and E. Petermann.** 2012. Replication fork dynamics and the DNA damage response. Biochem. J. **443**:13–26.
84. **Jurvansuu, J., M. Fragkos, C. Ingemarsdotter, and P. Beard.** 2007. Chk1 instability is coupled to mitotic cell death of p53-deficient cells in response to virus-induced DNA damage signaling. J Mol Biol **372**:397–406.
85. **Jurvansuu, J., K. Raj, A. Stasiak, and P. Beard.** 2004. Viral Transport of DNA Damage That Mimics a Stalled Replication Fork. J Virol **79**:569–580.
86. **Kashiwakura, Y., K. Tamayose, K. Iwabuchi, Y. Hirai, T. Shimada, K. Matsumoto, T. Nakamura, M. Watanabe, K. Oshimi, and H. Daida.** 2005.

Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection. J Virol **79**:609–614.
87. **Kastan, M. B., and D. S. Lim.** 2000. The many substrates and functions of ATM. Nature reviews **1**:179–186.
88. **Kawaguchi, Y., R. Bruni, and B. Roizman.** 1997. Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. J Virol **71**:1019–1024.
89. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J Virol **8**:125–132.
90. **Kim, D.-B., S. Zabierowski, and N. A. DeLuca.** 2002. The Initiator Element in a Herpes Simplex Virus Type 1 Late-Gene Promoter Enhances Activation by ICP4, Resulting in Abundant Late-Gene Expression. J Virol **76**:1548–1558.
91. **King, J. A.** 2001. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. The EMBO Journal **20**:3282–3291.
92. **Klinedinst, D. K., and M. D. Challberg.** 1994. Helicase-primase complex of herpes simplex virus type 1: a mutation in the UL52 subunit abolishes primase activity. J Virol **68**:3693–3701.
93. **Kobayashi, M., A. C. Wilson, M. V. Chao, and I. Mohr.** 2012. Control of viral latency in neurons by axonal mTOR signaling and the 4E-BP translation repressor. Genes & Development **26**:1527–1532.
94. **Kotin, R. M., J. C. Menninger, D. C. Ward, and K. I. Berns.** 1991. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics **10**:831–834.
95. **Kotin, R., M. Siniscalco, R. Samulski, X. Zhu, L. Hunter, C. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns.** 1990. Site-specific integration by adeno-associated virus. Proc Natl Acad Sci U S A **87**:2211–2215.
96. **Kyostio, S. R., R. S. Wonderling, and R. A. Owens.** 1995. Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. J Virol **69**:6787–6796.
97. **Lackner, D. F., and N. Muzyczka.** 2002. Studies of the mechanism of transactivation of the adeno-associated virus p19 promoter by Rep protein. J Virol **76**:8225–8235.
98. **Lafferty, W. E., R. W. Coombs, J. Benedetti, C. Critchlow, and L. Corey.** 1987. Recurrences after Oral and Genital Herpes Simplex Virus Infection. N Engl J Med **316**:1444–1449.
99. **Lavin, M. F., and S. Kozlov.** 2007. ATM activation and DNA damage response. Cell Cycle **6**:931–942.
100. **Lee, C. K., and D. M. Knipe.** 1985. An immunoassay for the study of DNA-binding activities of herpes simplex virus protein ICP8. J Virol **54**:731–738.
101. **Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J Virol **70**:7471–7477.
102. **Lees-Miller, S. P., K. Sakaguchi, S. J. Ullrich, E. Appella, and C. W. Anderson.** 1992. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. Molecular and cellular biology **12**:5041–5049.
103. **Leopardi, R., N. Michael, and B. Roizman.** 1995. Repression of the herpes simplex virus 1 alpha 4 gene by its gene product (ICP4) within the context of the

viral genome is conditioned by the distance and stereoaxial alignment of the ICP4 DNA binding site relative to the TATA box. J Virol **69**:3042–3048.
104. **Li, J., and D. F. Stern.** 2005. Regulation of CHK2 by DNA-dependent protein kinase. The Journal of biological chemistry **280**:12041–12050.
105. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A **102**:5844–5849.
106. **Lilley, C. E., M. S. Chaurushiya, C. Boutell, R. D. Everett, M. D. Weitzman, and J. D. Baines.** 2011. The Intrinsic Antiviral Defense to Incoming HSV-1 Genomes Includes Specific DNA Repair Proteins and Is Counteracted by the Viral Protein ICP0. PLoS Pathog **7**:e1002084.
107. **Lilley, C. E., R. A. Schwartz, and M. D. Weitzman.** 2007. Using or abusing: viruses and the cellular DNA damage response. Trends in Microbiology **15**:119–126.
108. **Liu, S., S. O. Opiyo, K. Manthey, J. G. Glanzer, A. K. Ashley, C. Amerin, K. Troksa, M. Shrivastav, J. A. Nickoloff, and G. G. Oakley.** 2012. Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. Nucleic Acids Research **40**:10780–10794.
109. **Loeffler, F. A., and P. Frosch.** 1898. Berichte der Kommission zur Erforschung der Maul- und Klauenseuche bei dem Institut für Infektionskrankheiten in Berlin. Cbl. Bakt. I. Abt., Orig:371–391.
110. **Long, M. C., V. Leong, P. A. Schaffer, C. A. Spencer, and S. A. Rice.** 1999. ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II. J Virol **73**:5593–5604.
111. **Maul, G. G., A. M. Ishov, and R. D. Everett.** 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology **217**:67–75.
112. **Mayor H.D., Jamison R.M., Jordan L.E., and Melnick J.L.** 1965. Structure and Composition of a Small Particle Prepared from a Simian Adenovirus. J Bacteriol. **90**:235–242.
113. **McCarty, D. M., S. M. Young, and R. J. Samulski.** 2004. Integration of Adeno-Associated Virus (AAV) and Recombinant AAV Vectors. Annu. Rev. Genet. **38**:819–845.
114. **McGregor, F., A. Phelan, J. Dunlop, and J. B. Clements.** 1996. Regulation of herpes simplex virus poly (A) site usage and the action of immediate-early protein IE63 in the early-late switch. J Virol **70**:1931–1940.
115. **Mohni, K. N., A. R. Dee, S. Smith, A. J. Schumacher, and S. K. Weller.** 2012. Efficient Herpes Simplex Virus 1 Replication Requires Cellular ATR Pathway Proteins. J Virol **87**:531–542.
116. **Mohni, K. N., C. M. Livingston, D. Cortez, and S. K. Weller.** 2010. ATR and ATRIP are recruited to herpes simplex virus type 1 replication compartments even though ATR signaling is disabled. J Virol **84**:12152–12164.
117. **Mohni, K. N., A. S. Mastrocola, P. Bai, S. K. Weller, and C. D. Heinen.** 2011. DNA Mismatch Repair Proteins Are Required for Efficient Herpes Simplex Virus 1 Replication. J Virol **85**:12241–12253.
118. **Mouw, M. B., and D. J. Pintel.** 2000. Adeno-associated virus RNAs appear in a temporal order and their splicing is stimulated during coinfection with adenovirus. J Virol **74**:9878–9888.

119. **Muralidhar, S., S. P. Becerra, and J. A. Rose.** 1994. Site-directed mutagenesis of adeno-associated virus type 2 structural protein initiation codons: effects on regulation of synthesis and biological activity. J Virol **68**:170–176.
120. **Muylaert, I., and P. Elias.** 2007. Knockdown of DNA Ligase IV/XRCC4 by RNA Interference Inhibits Herpes Simplex Virus Type I DNA Replication. Journal of Biological Chemistry **282**:10865–10872.
121. **Muzyczka, N., and Berns K.I** (ed.). 2001. Parvoviridae. The viruses and their replication. Fields Virology, Fourth ed., (D.M. Knipe and P.M. Howley, eds), Philadelphia.
122. **Nash, K., W. Chen, W. F. McDonald, X. Zhou, and N. Muzyczka.** 2007. Purification of Host Cell Enzymes Involved in Adeno-Associated Virus DNA Replication. J Virol **81**:5777–5787.
123. **Nash, K., W. Chen, M. Salganik, and N. Muzyczka.** 2008. Identification of Cellular Proteins That Interact with the Adeno-Associated Virus Rep Protein. J Virol **83**:454–469.
124. **Navaratnarajah, C. K., T. S. Miest, A. Carfi, and R. Cattaneo.** 2012. Targeted entry of enveloped viruses: measles and herpes simplex virus I. Current Opinion in Virology **2**:43–49.
125. **Newcomb, W. W., B. L. Trus, F. P. Booy, A. C. Steven, J. S. Wall, and J. C. Brown.** 1993. Structure of the herpes simplex virus capsid. Molecular composition of the pentons and the triplexes. J Mol Biol **232**:499–511.
126. **Nicola, A. V., A. M. McEvoy, and S. E. Straus.** 2003. Roles for Endocytosis and Low pH in Herpes Simplex Virus Entry into HeLa and Chinese Hamster Ovary Cells. J Virol **77**:5324–5332.
127. **Nicola, A. V., and S. E. Straus.** 2004. Cellular and Viral Requirements for Rapid Endocytic Entry of Herpes Simplex Virus. J Virol **78**:7508–7517.
128. **Nicolas, A., N. Alazard-Dany, C. Biollay, L. Arata, N. Jolinon, L. Kuhn, M. Ferro, S. K. Weller, A. L. Epstein, A. Salvetti, and A. Greco.** 2012. Identification of Rep-Associated Factors in Hsv-1-Induced Aav-2 Replication Compartments. J Virol.
129. **Nicolas, A., N. Jolinon, N. Alazard-Dany, V. Barateau, A. L. Epstein, A. Greco, H. Büning, and A. Salvetti.** 2012. Factors influencing helper-independent adeno-associated virus replication. Virology **432**:1–9.
130. **Nikitin, P. A., and M. A. Luftig.** 2011. At a crossroads: human DNA tumor viruses and the host DNA damage response. Future Virology **6**:813–830.
131. **Nimonkar, A. V., A. Z. Ozsoy, J. Genschel, P. Modrich, and S. C. Kowalczykowski.** 2008. Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. Proceedings of the National Academy of Sciences **105**:16906–16911.
132. **Ni, T. H., W. F. McDonald, I. Zolotukhin, T. Melendy, S. Waga, B. Stillman, and N. Muzyczka.** 1998. Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. J Virol **72**:2777–2787.
133. **Nony, P., J. Tessier, G. Chadeuf, P. Ward, A. Giraud, M. Dugast, R. M. Linden, P. Moullier, and A. Salvetti.** 2001. Novel cis-acting replication element in the adeno-associated virus type 2 genome is involved in amplification of integrated rep-cap sequences. J Virol **75**:9991–9994.
134. **Oliveira, A. P. de, and C. Fraefel.** 2010. Herpes Simplex Virus Type 1/Adeno-Associated Virus Hybrid Vectors~!2009-12-12~!2010-01-13~!2010-06-17~! TOVJ **4**:109–122.
135. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent

degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol **73**:650–657.
136. **Pellequer, J.-L., P. Parot, I. Liashkovich, W. Hafezi, J. M. Kühn, H. Oberleithner, and V. Shahin.** 2011. Nuclear delivery mechanism of herpes simplex virus type 1 genome. J. Mol. Recognit. **24**:414–421.
137. **Pereira D.J., and McCarty DM Muzyczka N.** 1997. The adeno-associated virus (AAV) Rep protein acts as both a repressor and an activator to regulate AAV transcription during a productive infection. J Virol **71**:1079–1088.
138. **Philpott, N. J.** 2002. A p5 integration efficiency element mediates Rep-dependent integration into AAVS1 at chromosome 19. Proceedings of the National Academy of Sciences **99**:12381–12385.
139. **Prasad, C., C. Meyers, D.-J. Zhan, H. You, M. Chiriva-Internati, J. L. Mehta, Y. Liu, and P. L. Hermonat.** 2003. The adeno-associated virus major regulatory protein Rep78-c-Jun-DNA motif complex modulates AP-1 activity. Virology **314**:423–431.
140. **Qing, K., C. Mah, J. Hansen, S. Zhou, V. Dwarki, and A. Srivastava.** 1999. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat Med **5**:71–77.
141. **Qiu J., and Pintel D.** 2008. Processing of adeno-associated virus RNA. Front Biosci. **1**:3101–3115.
142. **Raj, K., P. Ogston, and P. Beard.** 2001. Virus-mediated killing of cells that lack p53 activity. Nature **412**:914–917.
143. **Ray, S., and A. Almasan.** 2003. Apoptosis induction in prostate cancer cells and xenografts by combined treatment with Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand and CPT-11. Cancer Res **63**:4713–4723.
144. **Richardson, W. D., and H. Westphal.** 1981. A cascade of adenovirus early functions is required for expression of adeno-associated virus. Cell **27**:133–141.
145. **Roizman, B.** 2011. The Checkpoints of Viral Gene Expression in Productive and Latent Infection: the Role of the HDAC/CoREST/LSD1/REST Repressor Complex. J Virol **85**:7474–7482.
146. **Russell, D. W., I. E. Alexander, and A. D. Miller.** 1995. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. Proc Natl Acad Sci U S A **92**:5719–5723.
147. **Ruyechan, W. T., and A. C. Weir.** 1984. Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. J Virol **52**:727–733.
148. **Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter.** 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J **10**:3941–3950.
149. **Sanlioglu, S., M. M. Monick, G. Luleci, G. W. Hunninghake, and J. F. Engelhardt.** 2001. Rate limiting steps of AAV transduction and implications for human gene therapy. Curr Gene Ther **1**:137–147.
150. **Saudan, P., J. Vlach, and P. Beard.** 2000. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. The EMBO journal **19**:4351–4361.
151. **Schatz, D. G., and P. C. Swanson.** 2011. V(D)J Recombination: Mechanisms of Initiation. Annu. Rev. Genet. **45**:167–202.
152. **Schwartz, R. A., J. A. Palacios, G. D. Cassell, S. Adam, M. Giacca, and M. D. Weitzman.** 2007. The Mre11/Rad50/Nbs1 Complex Limits Adeno-Associated Virus Transduction and Replication. J Virol **81**:12936–12945.

153. **Sciabica, K. S., Q. J. Dai, and R. M. Sandri-Goldin.** 2003. ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation. EMBO J **22**:1608–1619.
154. **Sharma, S.** 2003. The Exonucleolytic and Endonucleolytic Cleavage Activities of Human Exonuclease 1 Are Stimulated by an Interaction with the Carboxyl-terminal Region of the Werner Syndrome Protein. Journal of Biological Chemistry **278**:23487–23496.
155. **Sheldrick, P., and N. Berthelot.** 1975. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor symposia on quantitative biology **39 Pt 2**:667–678.
156. **Shepard, A. A., P. Tolentino, and N. A. DeLuca.** 1990. trans-dominant inhibition of herpes simplex virus transcriptional regulatory protein ICP4 by heterodimer formation. J Virol **64**:3916–3926.
157. **Sherman, G., J. Gottlieb, and M. D. Challberg.** 1992. The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilization. J Virol **66**:4884–4892.
158. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. The Journal of biological chemistry **280**:30336–30341.
159. **Shrivastav, M., L. P. de Haro, and J. A. Nickoloff.** 2008. Regulation of DNA double-strand break repair pathway choice. Cell Res **18**:134–147.
160. **Sodeik, B.** 1997. Microtubule-mediated Transport of Incoming Herpes Simplex Virus 1Capsids to the Nucleus. The Journal of Cell Biology **136**:1007–1021.
161. **Song, S., Y. Lu, Y. K. Choi, Y. Han, Q. Tang, G. Zhao, K. I. Berns, and T. R. Flotte.** 2004. DNA-dependent PK inhibits adeno-associated virus DNA integration. Proceedings of the National Academy of Sciences of the United States of America **101**:2112–2116.
162. **Sonntag, F., K. Schmidt, and J. A. Kleinschmidt.** 2010. A viral assembly factor promotes AAV2 capsid formation in the nucleolus. Proceedings of the National Academy of Sciences **107**:10220–10225.
163. **Stanberry, L. R.** 2006. Neonatal herpes in premature infants: a special problem. Pediatrics **118**:2543–2544.
164. **Stracker, T. H., G. D. Cassell, P. Ward, Y.-M. Loo, B. van Breukelen, S. D. Carrington-Lawrence, R. K. Hamatake, P. C. van der Vliet, S. K. Weller, T. Melendy, and M. D. Weitzman.** 2004. The Rep Protein of Adeno-Associated Virus Type 2 Interacts with Single-Stranded DNA-Binding Proteins That Enhance Viral Replication. J Virol **78**:441–453.
165. **Summerford, C., and R. J. Samulski.** 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol **72**:1438–1445.
166. **Summers, K. C., F. Shen, E. A. Sierra Potchanant, E. A. Phipps, R. J. Hickey, and L. H. Malkas.** 2011. Phosphorylation: The Molecular Switch of Double-Strand Break Repair. International Journal of Proteomics **2011**:1–8.
167. **Surosky, R. T., M. Urabe, S. G. Godwin, S. A. McQuiston, G. J. Kurtzman, K. Ozawa, and G. Natsoulis.** 1997. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. J Virol **71**:7951–7959.
168. **Tattersall, P., and D. C. Ward.** 1976. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. Nature **263**:106–109.

169. **Tavalai, N., P. Papior, S. Rechter, M. Leis, and T. Stamminger.** 2006. Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J Virol **80**:8006–8018.
170. **Taylor, T. J., M. A. Brockman, E. E. McNamee, and D. M. Knipe.** 2002. Herpes simplex virus. Front Biosci **7**:d752-64.
171. **Taylor, T. J., and D. M. Knipe.** 2004. Proteomics of herpes simplex virus replication compartments. association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J Virol **78**:5856–5866.
172. **Tessier, J., G. Chadeuf, P. Nony, H. Avet-Loiseau, P. Moullier, and A. Salvetti.** 2001. Characterization of adenovirus-induced inverted terminal repeat-independent amplification of integrated adeno-associated virus rep-cap sequences. J Virol **75**:375–383.
173. **Tomimatsu, N., B. Mukherjee, and S. Burma.** 2009. Distinct roles of ATR and DNA-PKcs in triggering DNA damage responses in ATM-deficient cells. EMBO reports **10**:629–635.
174. **Turnell, A. S., and R. J. Grand.** 2012. DNA viruses and the cellular DNA-damage response. Journal of General Virology **93**:2076–2097.
175. **Umbach, J. L., M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen.** 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature.
176. **Vogel, R., M. Seyffert, R. Strasser, A. P. de Oliveira, C. Dresch, D. L. Glauser, N. Jolinon, A. Salvetti, M. D. Weitzman, M. Ackermann, and C. Fraefel.** 2011. Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection. J Virol **86**:143–155.
177. **Volcy, K., and N. W. Fraser.** 2013. DNA damage promotes herpes simplex virus-1 protein expression in a neuroblastoma cell line. J. Neurovirol. **19**:57–64.
178. **Walz, C., A. Deprez, T. Dupressoir, M. Durst, M. Rabreau, and J. R. Schlehofer.** 1997. Interaction of human papillomavirus type 16 and adeno-associated virus type 2 co-infecting human cervical epithelium. J Gen Virol **78 (Pt 6)**:1441–1452.
179. **Wanga, J., Faustb S.M., and Rabinowitz E.J.** 2011. The next step in gene delivery: Molecular engineering of adeno-associated virus serotypes. J Mol Cell Cardiol:793–802.
180. **Wang, S., M. Guo, H. Ouyang, X. Li, C. Cordon-Cardo, A. Kurimasa, D. J. Chen, Z. Fuks, C. C. Ling, and G. C. Li.** 2000. The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. Proceedings of the National Academy of Sciences of the United States of America **97**:1584–1588.
181. **Wang, X. S., S. Ponnazhagan, and A. Srivastava.** 1995. Rescue and replication signals of the adeno-associated virus 2 genome. J Mol Biol **250**:573–580.
182. **Wang, X. S., and A. Srivastava.** 1997. A novel terminal resolution-like site in the adeno-associated virus type 2 genome. J Virol **71**:1140–1146.
183. **Wang, Y., and W. Eckhart.** 1992. Phosphorylation sites in the amino-terminal region of mouse p53. Proceedings of the National Academy of Sciences of the United States of America **89**:4231–4235.
184. **Ward, P., M. Falkenberg, P. Elias, M. Weitzman, and R. M. Linden.** 2001. Rep-dependent initiation of adeno-associated virus type 2 DNA replication by a

herpes simplex virus type 1 replication complex in a reconstituted system. J Virol. **75**:10250–10258.
185. **Warrington, K., JR, O. Gorbatyuk, J. Harrison, S. Opie, S. Zolotukhin, and N. Muzyczka.** 2004. Adeno-Associated Virus Type 2 VP2 Capsid Protein Is Nonessential and Can Tolerate Large Peptide Insertions at Its N Terminus†. J Virol **78**:6595–6609.
186. **Weindler, F. W., and R. Heilbronn.** 1991. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. Journal of virology **65**:2476–2483.
187. **Weitzman, M. D., C. T. Carson, R. A. Schwartz, and C. E. Lilley.** 2004. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) **3**:1165–1173.
188. **Weitzman, M. D., K. J. Fisher, and J. M. Wilson.** 1996. Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. J Virol **70**:1845–1854.
189. **Weitzman, M. D., C. E. Lilley, and M. S. Chaurushiya.** 2010. Genomes in conflict. maintaining genome integrity during virus infection. Annual review of microbiology **64**:61–81.
190. **Weitzman, M. D., and R. M. Linden.** 2011. Adeno-Associated Virus Biology, p. 1–23. *In* R. O. Snyder and P. Moullier (ed.), Adeno-Associated Virus. Humana Press. Methods in molecular biology. Humana Press, Totowa, NJ.
191. **Weitzman, M. D., S. M. Young, T. Cathomen, and R. J. Samulski.** 1991. Targeted Integration by Adeno-Associated Virus. EMBO J **12**:201–220.
192. **Weller, S. K., and D. M. Coen.** 2012. Herpes Simplex Viruses: Mechanisms of DNA Replication. Cold Spring Harbor Perspectives in Biology **4**:a013011.
193. **Weston, M. C., H. Chen, and J. W. Swann.** 2012. Multiple Roles for Mammalian Target of Rapamycin Signaling in Both Glutamatergic and GABAergic Synaptic Transmission. Journal of Neuroscience **32**:11441–11452.
194. **Wilcock, D., and D. P. Lane.** 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature **349**:429–431.
195. **Wilkinson, D. E., and S. K. Weller.** 2003. The role of DNA recombination in herpes simplex virus DNA replication. IUBMB life **55**:451–458.
196. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of virology **78**:4783–4796.
197. **Wilkinson, D. E., and S. K. Weller.** 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. Journal of cell science **119**:2695–2703.
198. **Wilson, A. C., and I. Mohr.** 2012. A cultured affair: HSV latency and reactivation in neurons. Trends in Microbiology **20**:604–611.
199. **Winocour, E., M. F. Callaham, and E. Huberman.** 1988. Perturbation of the cell cycle by adeno-associated virus. Virology **167**:393–399.
200. **Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg.** 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J Virol **62**:435–443.
201. **Xiao, P.-J., and R. J. Samulski.** 2012. Cytoplasmic trafficking, endosomal escape, and perinuclear accumulation of adeno-associated virus type 2 particles are facilitated by microtubule network. J Virol **86**:10462–10473.

202. **Xiao, W., N. Chirmule, S. C. Berta, B. McCullough, G. Gao, and J. M. Wilson.** 1999. Gene therapy vectors based on adeno-associated virus type 1. J Virol **73**:3994–4003.
203. **Xiao, W., K. H. Warrington, P. Hearing, J. Hughes, and N. Muzyczka.** 2002. Adenovirus-Facilitated Nuclear Translocation of Adeno-Associated Virus Type 2. Journal of Virology **76**:11505–11517.
204. **Yakobson, B., T. A. Hrynko, M. J. Peak, and E. Winocour.** 1989. Replication of adeno-associated virus in cells irradiated with UV light at 254 nm. J Virol **63**:1023–1030.
205. **Yakobson, B., T. Koch, and E. Winocour.** 1987. Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. J Virol **61**:972–981.
206. **Yalkinoglu, A. O., R. Heilbronn, A. Burkle, J. R. Schlehofer, and H. Zur Hausen.** 1988. DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. Cancer Res **48**:3123–3129.
207. **Young, R. C.** 2010. Incidence, Recurrence, and Outcomes of Herpes Simplex Virus Eye Disease in Olmsted County, Minnesota, 1976-2007The Effect of Oral Antiviral ProphylaxisEpidemiology of HSV and Oral Antivirals. Arch Ophthalmol. **128**:1178–1183.
208. **Zaitlin, M.** (1998). The Discovery of the Causal Agent of the Tobacco Mosaic Virus Disease, p. 105–110. *In* S. D. Kung and S. F. Yang (ed.), Discoveries in plant biology. World Scientific Publ. Co. World Scientific Publ. Co., Singapore [u.a.].
209. **Zhou, Z. H., B. V. Prasad, J. Jakana, F. J. Rixon, and W. Chiu.** 1994. Protein subunit structures in the herpes simplex virus A-capsid determined from 400 kV spot-scan electron cryomicroscopy. J Mol Biol **242**:456–469.

## 3.6 Viral and cellular components of AAV2 replication compartments.

I have written a review article for submission to the *Open Virology Journal*, which analyzes and discusses the known interactions of helpervirus and cellular proteins with AAV2 Rep proteins or AAV2 RCs. I include this manuscript here as part of the Introduction of my thesis*. My own contribution includes: literature search and analysis and writing of the manuscript. I received editorial help from the following co-authors: Michael Seyffert, Bruna de Andrade Pereira, and Cornel Fraefel.*

*Send Orders for Reprints to reprints@benthamscience.net*





# Viral and Cellular Components of AAV2 Replication Compartments

Rebecca Vogel, Michael Seyffert, Bruna de Andrade Pereira and Cornel Fraefel*

*Institute of Virology, University of Zurich, Winterthurerstr. 266a, CH-8057 Zurich, Switzerland*

**Abstract:** Adeno-associated virus 2 (AAV2) is a helpervirus-dependent parvovirus with a bi-phasic life cycle comprising latency in absence and lytic replication in presence of a helpervirus, such as adenovirus (Ad) or herpes simplex virus type 1 (HSV-1). Helpervirus-supported AAV2 replication takes place in replication compartments (RCs) in the cell nucleus where virus DNA replication and transcription occur. RCs consist of a defined set of helper virus-, AAV2-, and cellular proteins. Here we compare the profile of cellular proteins recruited into AAV2 RCs or identified in Rep78-associated complexes when either Ad or HSV-1 is the helpervirus, and we discuss the potential roles of some of these proteins in AAV2 and helpervirus infection.

**Keywords:** AAV2, HSV-1, adenovirus, replication compartments.

## ADENO-ASSOCIATED VIRUS - BIOLOGICAL PROPERTIES

AAV2 is one of the most promising vectors for human gene therapy [1]. The apparent lack of pathogenicity and low immunogenicity together with the capability to infect both dividing and non-dividing cells constitute perfect characteristics for a transgene delivery vector. AAV2 is a small, icosahedral and non-enveloped particle, which has a size of about 20-22 nm in diameter. Its genome consists of a linear single-stranded DNA with a size of 4.7kb and encodes two open reading frames (ORF), rep and cap. The rep ORF is regulated by two promoters (p5 and p19) which in cooperation with a common splicing site encode four Rep proteins termed Rep40, Rep52, Rep68 and Rep78, named according to their apparent molecular weight. The two large Rep proteins Rep68 and Rep78 are required for AAV2 DNA replication, self-regulation of transcription, site-specific integration as well as for inhibition of helpervirus replication, whereas the smaller Rep40 and Rep52 are believed to be required for packaging [2-4]. The cap ORF is controlled by the p40 promoter and encodes the three structural proteins VP1, VP2 and VP3 [5,6]. Moreover, a nested alternative ORF of the cap gene has been shown recently to encode a protein, designated assembly-activating protein (AAP), which is believed to be required for AAV2 capsid assembly in the nucleolus [7].

AAV2 belongs to the family of the Parvoviridae and the genus Dependovirus, as its replication depends on the simultaneous infection with a helper virus, such as Ad, HSV-1, or papillomavirus [8-12]. In absence of a helper virus however, AAV2 establishes a latent infection, either by maintaining its genome in an episomal state [13] or integrating it into a well-defined locus termed AAVS1 on human chromosome 19 (Ch19) at position 19q13.4 [14-18]. Upon infection with a helpervirus, AAV2 is rescued from latency and enters a lytic life cycle in which the viral DNA is replicated and progeny virus is produced.

The AAV2 DNA has a unique structure. Its two ends are characterized by 145 nt palindromic sequences termed inverted terminal repeats (ITR) which fold into hairpins. Just as unique as the AAV2 DNA structure is the rolling-hairpin mechanism of AAV2 DNA replication: the end of the 3'-ITR acts as the essential primer for second-strand synthesis, a process termed self-priming. The following asymmetric leading-strand DNA synthesis resembles a rolling-circle model of DNA replication, resulting in a closed-end intermediate at the 3'-ITR. This structure is resolved by the so called terminal resolution step, which involves a site- and strand-specific endonuclease cut performed by Rep68/78 at the so called terminal resolution site (TRS; [19-21]). The following unwinding of the remaining 3'-ITR allows to complete the replication process.

## ADENOVIRUS AND HERPES SIMPLEX VIRUS TYPE 1 - BIOLOGICAL PROPERTIES

Adenovirus is the best characterized helper virus for AAV2. Adenoviruses are non-enveloped, double-stranded DNA viruses. Their capsid is characterized by a well-defined icosahedral structure consisting of three major proteins, hexon (II), penton base (III) and long knobbed projection termed fiber (IV), which is required to bind to the cell surface adenoviral receptor (CAR). The linear 26-45 kb dsDNA has inverted terminal repeats (ITRs) at each end, where a terminal protein (TP) is covalently attached to it [22]. The genome encodes four early transcription cassettes termed E1 (E1A and E1B), E2, E3 and E4, and five late gene products designated L1 to L5 which result from a series of splicing events of the major late (ML) transcript and constitute the structural proteins.

The adenovirus life cycle can be divided into two phases. The early phase is determined as the entry of the particle into the cell and the trafficking of the viral genome to the nucleus where expression of the early genes occurs. The second, or

*Address correspondence to this author at the Institute of Virology, University of Zurich, Winterthurerstr. 266a, CH-8057 Zurich, Switrzerland; Tel: +41 44 635 8713; Fax: +41 44 635 8911;
E-mail: cornel.fraefel@access.uzh.ch

1874-3579/13 

late, phase includes the virus DNA replication and the expression of late genes, followed by virus assembly and egress from the nucleus and the cell.

HSV-1 is another well characterized and efficient helper virus for AAV2 replication. It is a widespread human pathogen which has also both a lytic and a latent phase. It infects mainly mucosal tissue and neurons. The viral particle is composed of three structural compartments, (i) the viral envelope, (ii) the tegument consisting of a distinct set of viral proteins and (iii) the capsid which encloses the viral genome. The HSV-1 genome is a double-stranded DNA of 152 kbp which encodes at least 84 proteins.

HSV-1 can enter the cell both by fusion at the cell membrane or endocytosis and fusion with the endosomal membrane. The capsid is transported along microtubules to the nuclear pores, where the HSV-1 DNA, along with specific HSV-1 tegument proteins such as VP16 and the virion host shut-off protein, enters the nucleus. VP16 then transactivates the expression of the viral immediate early (IE) genes *via* the cellular RNA polymerase II [23]. The IE proteins have regulatory functions and activate the expression of the early (E) genes. Many of the E gene products are enzymes involved in DNA metabolism and their synthesis initiates the replication of the HSV-1 genome. The IE gene products and the replication of the HSV-1 DNA then activate expression of the late (L) genes, which encode structural components of the virion. HSV-1 can also establish latent infections, primarily in sensory neurons. During latency, HSV-1 gene expression is limited to the latency-associated transcripts (LATs) and no viral proteins are synthesized [24]. The mechanisms that control productive or latent infection of HSV-1 are not fully understood.

## THE NUCLEAR REPLICATION COMPARTMENTS OF DNA VIRUSES

### Viral RCs Develop Adjacent to PML Bodies

Similar to many other DNA viruses including Ad and HSV-1, AAV2 DNA replication takes place in so called viral replication compartments (RCs) or replication centers in the host cell nucleus [25-29]. The cell nucleus is a highly structured and compartmentalized space, containing not only the genome but also a multitude of proteins organized in subnuclear organelles [30]. Several of these nuclear bodies including clastosomes, nuclear speckles, nucleoli, or promyelocytic leukemia (PML) bodies contain proteins involved in central cellular processes such as proteolysis, transcription, apoptosis, or DNA-damage sensing and repair [31]. It seems to be a general feature of nuclear replicating DNA viruses that incoming viral genomes initially associate with PML bodies (also termed as ND10 nuclear bodies) and that the viral replication centers mature juxtaposed to these nuclear subcompartments [32-34]. PML bodies are functionally complex, containing proteins not only involved in DNA replication, transcription, or epigenetic silencing but also in the host defense mechanisms against viral infection [35]. It seems that viruses have evolved strategies to exploit PML bodies in order to initiate viral gene transcription and viral DNA replication [32,36-39]. For that reason, the often observed initial association of incoming viral genomes with PML bodies [32-34] might be a consequence of the cellular front line defense against viruses, however efficiently hijacked by the virus. During Ad supported AAV2 replication, AAV2 DNA has been found associated also with PML bodies [25], similar to cells infected with Ad alone [37,38], and AAV2 RCs mature juxtapose to PML bodies [25]. With the onset of infection, several replication and repair proteins, normally localized to PML bodies, have been found associated with Ad supported AAV2 RCs (discussed below). The HSV-1 ICP0 protein can efficiently disrupt PML bodies [40], and indeed, HSV-1-supported AAV2 RCs did not co-exist with PML bodies [25]. Nevertheless, integral cellular PML bodies might have an effect on initial AAV2 gene expression as well as the composition of cellular proteins in AAV2 RCs, as similar to Ad coinfection, several cellular replication and repair factors of PML bodies accumulate also in HSV-1 supported AAV2 RCs (discussed below).

### Spatial and Temporal Organization of RCs

Viral RCs are composed of a multitude of cellular proteins. These factors may alter depending on the necessity of the different viruses but also depending on the different functions of viral RCs, which may change spatially and temporally over the course of replication. In HSV-1 infected cells, besides viral DNA and RNA metabolism, capsid assembly and genome packaging is also localized to viral RCs [41-43]. By contrast, Ad DNA replication and assembly is suggested to occur in distinct nuclear compartments [44]. AAV2 capsid assembly has been observed to occur also dislocated from viral RCs in nucleoli [45]. However, the site of genome packaging into pre-assembled capsids remains to be identified.

In the host cell, high transcriptional activity in G1 phase [46] is separated in a time dependent manner from genome replication in S-phase of the cell cycle. In contrast, viruses have to create an environment which enables high transcriptional activity of viral genes as well as the production of viral DNA for progeny virions in parallel. Therefore, viral RCs have to be well structured microenvironments not only in a temporal but also in a spatial manner. For example, Ad RCs are surrounded by ring-like structures called peripheral replicative zones, where Ad DNA replication transcription and pre-mRNA processing occurs [47,48]. The defined pattern of VP5, ICP4, and ICP8 within HSV-1 RCs [41] also indicates a complex organization. In contrast, besides the well described vast growth dynamics of AAV2 RCs [25], not much is known about their spatial and temporal organization. The identification of factors associated with AAV2 RCs, as well as the definition of their localization may help to better understand the organization of helper supported AAV2 replication.

## HSV-1 & AD HELPER VIRUS PROTEINS INVOLVED IN AAV2 REPLICATION

In addition to multiple cellular proteins, a defined set of helper virus proteins are involved in AAV2 replication. The best studied helper virus proteins for AAV2 replication are provided by Ad and HSV-1. In case of Ad, these helper factors include E1A, E1B55K, E2A, E4orf6, and the VA RNA [49-51]. The Ad E1A protein not only promotes transcription of the early Ad genes, but also transcription of

the AAV2 Rep ORF [52]. E1B55K and E4orf6 form a heterodimer that supports AAV2 mRNA export from the nucleus [53], and the VA RNAs play a central role in AAV2 infection by maintaining protein translation [54]. The DNA-binding protein E2A supports multiple steps of the AAV2 life cycle including regulation of AAV2 gene expression, viral mRNA processing and export, and viral DNA replication [55,56].

The minimal set of HSV-1 helper proteins required for AAV2 replication includes the helicase-primase complex encoded by UL5, UL8, and UL52 and the major DNA binding protein ICP8 (UL29; [57-59]). All of these proteins are also mandatory for HSV-1 replication [60]. In addition, the HSV-1 origin binding protein (UL9), the viral DNA polymerase (UL30) and the ds DNA binding protein UL42 have been found to support AAV2 replication [57,58]. It has been shown that the UL5 helicase activity is necessary for efficient AAV2 DNA replication, whereas the primase-activity of UL52 is redundant [61]. Although not much is known about the detailed function of ICP8 in AAV2 replication, it seems that it is linked to the interaction with AAV2 Rep78, in an AAV2-ss DNA dependent manner [58,59,62].

The minimal set of HSV-1 helper proteins (UL5, UL8, UL29, UL52) together with two other HSV-1 proteins, the viral DNA polymerase (UL30) and the ds DNA binding protein UL42, along with AAV2 Rep68 have been shown to be also sufficient to initiate replication of duplex DNA, containing the AAV2 origins of DNA replication, in an *in vitro* replication system [59].

## CELLULAR PROTEINS ASSOCIATED WITH HSV-1 OR AD SUPPORTED AAV2 RCs

While helper virus proteins involved in AAV2 replication are well studied [63], not much is known about the role of host proteins recruited to AAV2 RCs. Nevertheless, these proteins are likely implicated in several steps of the AAV2 life cycle including AAV2 DNA replication, gene expression and posttranscriptional modification. The recruitment of host cell proteins into AAV2 RCs may alter, depending on the type of helper virus. However, it is likely that AAV2 might also recruit a set of indispensable cellular proteins, independent of the type of helper virus.

In order to identify host proteins associated with AAV2 replication, co-immunoprecipitation (co-IP) assays of AAV2 Rep78 and immunofluorescence analysis in presence or absence of a helper virus have been performed (Tables **1** and **2**; [64-73]). However, the majority of proteins were identified only by mass spectrometry of Rep78-associated complexes [64,68], and many of these interactions remain to be validated. Nevertheless, our aim was to analyze these cellular proteins concerning similarities and differences. We used the string database (http://string-db.org/) to reconstitute possible interaction networks between identified cellular proteins associated with AAV2 RCs in presence of Ad (Table **1**, Fig. **1**) or HSV-1 (Table **2**, Fig. **2**). The database lists confident known and predicted direct (physical) and indirect (functional) interactions between cellular proteins, based on experimental validations.

Based on the string database, the two main groups of cellular proteins of HSV-1 supported AAV2 RCs include DNA replication and repair proteins, and RNA splicing factors (Fig. **2**). Similar to HSV-1 coinfection, a governing group of proteins associated with Ad supported AAV2 RCs belong to the cellular DNA replication and repair machinery (Fig. **1**). In addition, a multitude of transcriptional regulators as well as RNA splicing factors and a noticeable network of mRNA and protein trafficking factors were identified in AAV2 and Ad coinfected cells (Fig. **1**). Besides these main groups, also proteins involved in nuclear organization, translation, protein degradation, cytoplasmic signaling, and many more were identified in AAV2 Rep78 co-IP assays of cells coinfected with AAV2 and either HSV-1 or Ad as the helpervirus. In the following, we discuss a selection of these proteins in more detail including their potential functions in different steps of the AAV2 life cycle.

## PROTEINS INVOLVED IN AAV2 DNA AND RNA METABOLISM

### Cellular Proteins Promoting Initial AAV2 Second-Strand Synthesis and AAV2 DNA Replication

Due to the ss nature of the AAV2 genome, AAV2 replication initially requires DNA second-strand synthesis before viral genes can be expressed and viral DNA replication can occur. The formation of ds genomes is a critical step for wtAAV2 to initiate productive infection, but also for rAAV2 vectors, as this step is of importance for successful expression of transgene sequences [74]. Second-strand synthesis is believed to be mediated through the host replication machinery, but helper virus proteins might be involved as well [75].

Prior to polymerase-mediated displacement of the parental DNA strand, a pre-replication complex is formed, which loads the whole DNA replication machinery onto the DNA [76]. The MCM proteins 2-7 are essential components of this complex [76]. Several of these proteins were found in both Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**) and have been also found to be required to replicate AAV2 DNA from a ssDNA template in an *in vitro* replication assay [77,78]. Upon initial second-strand synthesis MCM might participate in the formation of a replication complex on the 3' end of the incoming ss AAV2 DNA template. The complementary self-annealing sequence of the AAV2 ITR provides a base-paired 3' hydroxyl group for initial unidirectional DNA synthesis [75]. Because AAV2 provides its own DNA primer, viral DNA replication exclusively uses leading-strand DNA synthesis and not lagging-strand synthesis. Although both helper viruses, Ad and HSV-1, express their own polymerase, AAV2 seems to use only the HSV-1 polymerase [57], while the Ad DNA polymerase has not been found to be involved in AAV2 DNA replication [49-51,79]. In contrast to AAV2 replication, Ad DNA synthesis uses a protein priming mechanism in which the viral polymerase (AdPol) recognizes and binds to the viral preterminal protein (pTP); the pTP-dCMP complex then serves as the primer for subsequent elongation catalyzed by AdPol *via* a strand displacement mechanism. Since the AdPol needs to form a stable heterodimer with pTP to initiate its DNA replication *via* a protein priming mechanism, and AAV2 replication uses a DNA primer to initiate viral DNA replication, it is not surprising that the Ad polymerase does not contribute to

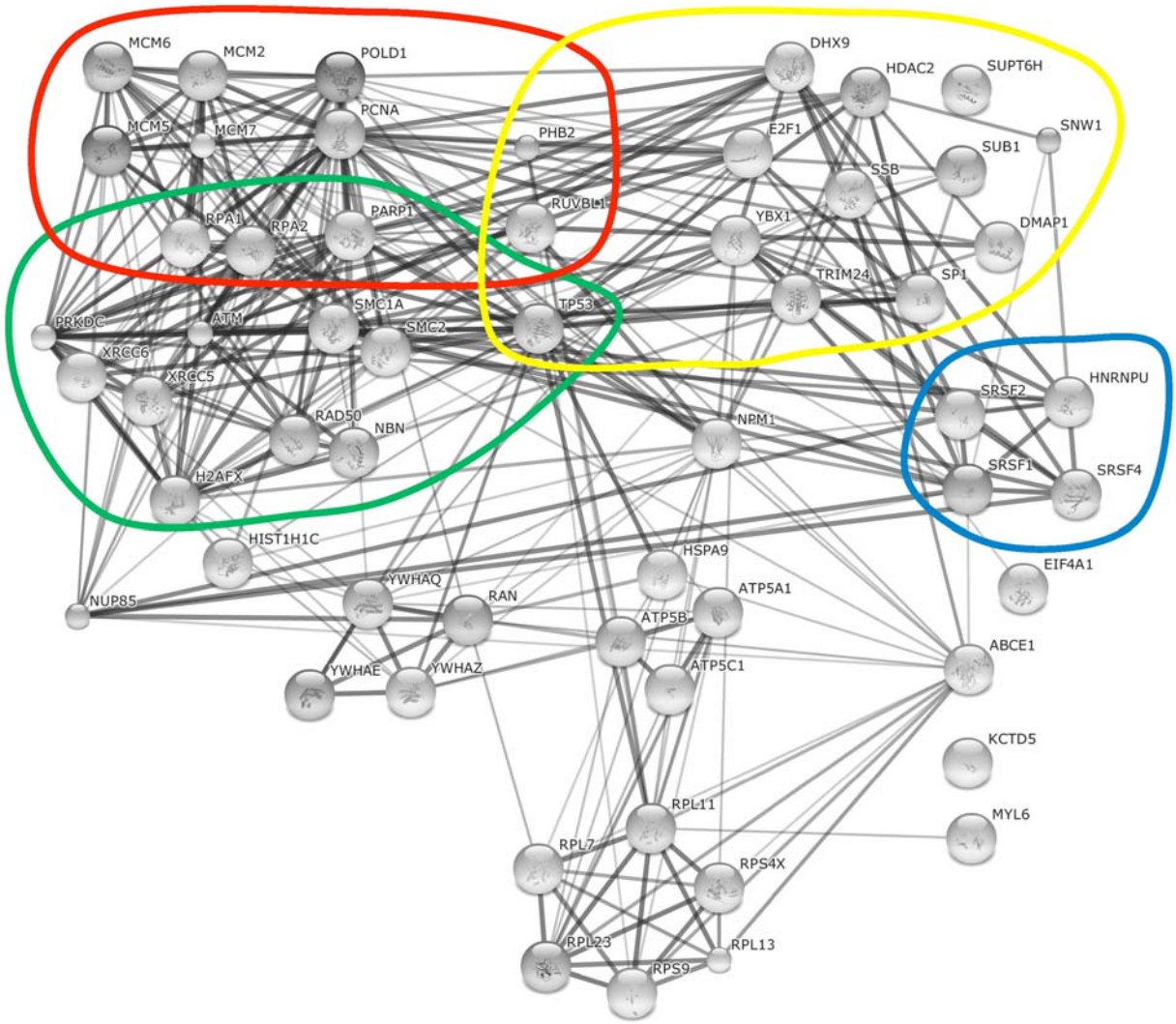



**Fig. (1).** Cellular proteins associated with Ad supported AAV2 RCs. The String database (http://string-db.org/) was used to reconstitute the interaction networks between identified cellular proteins associated with Ad supported AAV2 RCs and/or the AAV2 Rep78 protein. The database lists confident known and predicted direct (physical) and indirect (functional) interactions between cellular proteins based on experimental validations. Four functional categories, mRNA processing (blue), DNA replication (red), transcription (yellow), and DNA damage sensing and repair (green) are highlighted.

AAV2 DNA replication. HSV-1 replication is initiated by binding of the UL9 protein and unwinding of the HSV-1 *ori* [80]. Subsequently, the viral polymerase catalyzes DNA synthesis at the leading-strand and lagging-strand. At the eukaryotic replication fork, DNA polymerase delta (POLD) together with PCNA is suggested to build the lagging-strand replisome, and polymerase epsilon together with PCNA the leading-strand replisome [81]. However, PCNA together with POLD1 was found associated with AAV2-Rep78 during coinfection with Ad (Table **1**, Fig. **1**) and therefore might be the essential polymerase for leading-strand synthesis of the viral DNA. In addition, chromatin immunoprecipitation (ChIP) studies revealed that DNA POLD, but not polymerase alpha and epsilon, directly binds to UV inactivated AAV2 genomes, independent of the presence of a helper virus [82]. Collectively, these findings are in line with the observation that upon SV40 infection, POLD is also responsible for leading-strand synthesis of the viral DNA [83]. Interestingly, PCNA and POLD1 were also found in HSV-1 supported AAV2 RCs (Table **2**, Fig. **2**), although the HSV-1 polymerase complex has been shown to be sufficient to promote productive AAV2 infection [57,58]. Second-strand synthesis of the ssAAV2 genome leads to the formation of a duplex DNA molecule that is covalently closed at one end by the hairpin structure of the ITR initially used as a DNA primer. In order to complete replication, in a next step called terminal resolution, the remaining hairpin has to be resolved and replicated to yield a linear ds DNA [75]. For this, the AAV2 Rep protein binds to the RBS motif in order to promote cleavage of one strand at the unique TRS. The regenerated 3'hydroxyl group within the ITR then provides the basis for the replication through the viral ITR [75]. In a last step called reinitiation, a double-hairpinned intermediate is formed by denaturation and reannealing of the linear ITR [75]. This last step initiates a new round of strand displacement synthesis, generating a ss AAV2 genome that can be packaged into a capsid [75].

**Table 1. Proteins Identified in Rep78-Associated Complexes and/or AAV2 RCs During Ad Supported AAV2 Replication**

| Protein Name | Gene Name/ Abbreviation | Alternative Names/ Abbreviations | UniProt ID | Reference |
|---|---|---|---|---|
| ATP-binding cassette subfamily D member 3 | ABCD3 | (PMP70, PXMP1) | P28288 | [64] |
| ATP-binding cassette subfamily E member 1 | **ABCE1** | (RLI, RNASEL1, RNS4I) | P61221 | [64] |
| A-kinase anchor protein 12 | AKAP12 | (AKAP250) | Q02952 | [64] |
| ADP-ribosylation factor 1 | ARF1 | | P84077 | [64] |
| Coatomer ADP-ribosylation factor 4 | ARF4 | (ARF2) | P18085 | [64] |
| Serine-protein kinase ATM | **ATM** | (A-T mutated) | Q13315 | [65-66] |
| Sodium/potassium-transporting ATPase α | ATP1A1 | | P05023 | [64] |
| SERCA2A | ATP2A2 | (ATP2B) | P16615 | [64] |
| ATP synthase subunit alpha, mitochondrial | **ATP5A1** | (ATP5AL2, ATPM ) | P25705 | [64] |
| ATP synthase subunit beta, mitochondrial | **ATP5B** | (ATPMB, ATPSB) | P06576 | [64] |
| ATP synthase subunit gamma, mitochondrial | **ATP5C1** | (ATP5CL1, ATPG) | P36542 | [64] |
| ELG protein variant (fragment) | C17orf85 | (ELG) | Q53F19 | [64] |
| Calcium-dependent protein kinase type II δ | CAMK2D | | Q4G1A8 | [64] |
| Calcium-dependent protein kinase type II γ | CAMK2G | | Q8WU40 | [64] |
| Coatomer subunit α | COPA | (HEPCOP) | P53621 | [64] |
| Aspartyl-tRNA synthetase | DARS | DARS | Q53T60 | [64] |
| Dolichyl-diphosphooligosaccharide transferase | DDOST | (KIAA0115, OST48) | P39656 | [64] |
| DEAH (Asp-Glu-Ala-His) box polypeptide 9 | **DHX9** | | Q6PJK6 | [64] |
| DNA methyltransferase 1-associated protein 1 | **DMAP1** | (KIAA1425) | Q9NPF5 | [64] |
| 14-3-3 Dedicator of cytokinesis protein 7 | DOCK7 | | A4FU72 | [64] |
| Dynein heavy chain, cytosolic | DYNC1H1 | | Q6P2H7 | [64] |
| Transcription factor E2F1 | **E2F1** | (RBBP3) | Q01094 | [69] |
| Elongation factor 1-γ | EEF1G | | Q53YD7 | [64] |
| Elongation factor 2 | EEF2 | | Q6PK56 | [64] |
| Eukaryotic initiation factor 4A-I | EIF4A1 | (DDX2A, **eIF4FA1** ) | P60842 | [64] |
| RNA-binding protein FUS | FUS | (TLS) | P35637 | [64] |
| Histone H2A.x | H2AFX | (H2AX, H2a/x) | P16104 | [65] |
| Histone deacetylase 2 variant (fragment) | **HDAC2** | (HD2) | Q92769 | [64] |
| HINT4 (histidine triad protein 3) | HINT3 | | Q9NQE9 | [64] |
| Histone H1.2 | HIST1H1C | (H1F2) | P16403 | [64] |
| Heterogenous nuclear ribonucleoprotein U | HNRNPU | (**hnRNPU**, SAFA, U21.1) | Q00839 | [64] |
| Heat shock cognate 71 kDa protein | HSPA8 | (HSC70, HSP73, HSPA10) | P11142 | [64] |
| Stress-70 protein, mitochondrial | **HSPA9** | (GRP75, HSPA9B, mt-HSP70) | P38646 | [64] |
| Interleukin enhancer-binding factor 3 | ILF3 | (DRBF, MPHOSPH4, NF90) | Q12906 | [64] |
| Importin-7 | IPO7 | (RANBP7) | O95373 | [64] |
| Insulin receptor substrate 4 | IRS4 | (py160, pp160) | O14654 | [64] |
| BTB/POZ domain-containing protein KCTD5 | **KCTD5** | | Q9NXV2 | [73] |
| KH-type splicing regulatory protein | KHSRP | (FUBP2) | Q92945 | [64] |
| Kinesin 1 heavy chain | KIF5B | | Q6P164 | [64] |
| Kinesin light chain 2 | KLC2 | | Q9H0B6 | [64] |
| BC002942 protein | LMF2 | (TMEM112B, TMEM153) | Q9BU23 | [64] |

**(Table 1) contd…..**

| Protein Name | Gene Name/ Abbreviation | Alternative Names/ Abbreviations | UniProt ID | Reference |
|---|---|---|---|---|
| Lysozyme C precursor | LYZ | (LZM) | P61626 | [64] |
| DNA replication licensing factor MCM3 | **MCM2** | (BM28, CCNL1, CDCL1, KIAA0030) | P49736 | [64] |
| DNA replication licensing factor MCM5 | **MCM5** | (CDC46) | P33992 | [64] |
| DNA replication licensing factor MCM6 | **MCM6** | (p105MCM) | Q14566 | [64] |
| DNA replication licensing factor MCM7 | **MCM7** | (CDC47) | P33993 | [64] |
| Myosin light polypeptide 6 | MYL6 | (MYL6, LC17, MLC3) | P60660 | [64] |
| Nibrin | NBN | (NBS1, P95) | O60934 | [66] |
| Nucleolin | NCL | | P19338 | [64] |
| Nucleophosmin | **NPM1** | (Nucleolar phosphoprotein B23) | P06748 | [64],[70] |
| Nucleoporin 85 | NUP85 | (NUP75, PCNT1) | Q9BW27 | [64] |
| Poly-[ADP-ribose] polymerase 1 | PARP1 | (ADPRT, PPOL) | P09874 | [64] |
| Proliferating cell nuclear antigen | PCNA | | P12004 | [64] |
| Prohibitin-2 | PHB2 | (BAP, REA) | Q99623 | [64] |
| DNA polymerase delta catalytic subunit | POLD1 | | P28340 | [64] |
| Protein phosphatase 1 regulatory subunit 26 | PPP1R26 | (KIAA0649) | Q5T8A7 | [64] |
| DNA-dependent protein kinase | PRKDC | (HYRC1, DNA-PKcs) | P78527 | [64-66] |
| U4/U6 small nuclear ribonucleoprotein Prp4 | PRPF4 | | Q6IAP9 | [64] |
| 26S protease regulatory subunit 7 | PSMC2 | | Q75L23 | [64] |
| Proteasome non-ATPase regulatory subunit 2 | PSMD2 | (TRAP2) | Q13200 | [64] |
| DNA repair protein RAD50 | RAD50 | | Q92878 | [64] |
| GTP-binding nuclear protein RAN | **RAN** | (ARA24) | P62826 | [64] |
| E3 SUMO-protein ligase RanBP2 | RANBP2 | (NUP358) | P49792 | [64] |
| Reticulocalbin-1 precursor | RCN1 | | Q15293 | [64] |
| Telomere-associated protein RIF1 | RIF1 | | Q5UIP0 | [64] |
| Replication protein A 70 kDa DNA-binding subunit | RPA1 | (REPA1, **RPA70**) | P27694 | [64] |
| 32-kDa replication protein A | RPA2 | (REPA2, **RPA32**, RPA34) | P15927 | [64-65] |
| 60S ribosomal protein L11 | RPL11 | | P62913 | [64] |
| 60S ribosomal protein L13 | RPL13 | (BBC1) | P26373 | [64] |
| 60S ribosomal protein L23 | RPL23 | (RPL23 A) | P62829 | [64] |
| 60S ribosomal protein L7 | RPL7 | (RPL7 A) | P18124 | [64] |
| RPS4X protein | RPS4X | | Q96IR1 | [64] |
| RPS9 protein | RPS9 | | A5D904 | [64] |
| RuvB-like 1 | **Ruvbl1** | (INO80H, NMP238, TIP49, TIP49A) | Q9Y265 | [64] |
| Splicing factor 3B subunit 4 | **SF3B4** | SAP49 | Q15427 | [64] |
| Mitochondrial 2-oxoglutarate/malate carrier protein | SLC25A11 | (SLC20A4, OGCP) | Q02978 | [64] |
| Phosphate carrier protein, mitochondrial | SLC25A3 | (PHC, PTP) | Q00325 | [64] |
| SLC25A5 protein | SLC25A5 | (SLC25A5) | Q6NVC0 | [64] |
| ADP/ATP translocase 3 | SLC25A6 | (ANT3) | P12236 | [64] |
| Structural maintenance of chromosomes protein 1A | SMC1A | (DXS423E, KIAA0178, SB1.8, **SMC1**, SMC1L1) | Q14683 | [65] |
| Structural maintenance of chromosome 2 | **SMC2** | (CAPE, SMC2L1) | O95347 | [64] |
| SNW domain-containing protein 1 | **SNW1** | | Q0D2M5 | [64] |

**(Table 1) contd…..**

| Protein Name | Gene Name/ Abbreviation | Alternative Names/ Abbreviations | UniProt ID | Reference |
|---|---|---|---|---|
| Transcription factor Sp1 | **SP1** | (TSFP1) | P08047 | [71] |
| Spectrin β chain, brain 2 | SPTBN2 | | A4QPE4 | [64] |
| Splicing coactivator subunit SRm300 | **SRRM2** | | Q05BI2 | [64] |
| Splicing factor, arginine/serine-rich 1 | **SRSF1** | (ASF, SF2, SF2P33) | Q07955 | [64] |
| Splicing factor, arginine/serine-rich 2 | **SRSF2** | | Q01130 | [64] |
| Splicing factor, arginine/serine-rich 4 | **SRSF4** | (SRP75) | Q08170 | [64] |
| Lupus La protein | **SSB** | | P05455 | [64] |
| Stomatin-like protein 2, mitochondrial | STOML2 | (SLP2) | Q9UJZ1 | [64] |
| RNA polymerase II coactivator p15 (PC4) | **SUB1** | (PC4, RPO2TC1) | P53999 | [64] |
| Arginine/serine-rich-splicing factor 14 | SUGP2 | (KIAA0365, SFRS14) | Q8IX01 | [64] |
| Transcription elongation factor SPT6 | **SUPT6H** | (KIAA0162, SPT6H) | Q7KZ85 | [64] |
| p53 | TP53 | (**p53**) | P04637 | [72] |
| Transcription intermediary factor 1-α | **TRIM24** | (RNF82, TIF1, TIF1A) | O15164 | [64] |
| Elongation factor Tu, mitochondrial | TUFM | (P43, EFTU ) | P49411 | [64] |
| Splicing factor U2AF 35-kDa subunit | U2AF1 | (U2AF35, U2AFBP) | Q01081 | [64] |
| Ubiquitin-activating enzyme E1 | UBA1 | (A1S9T, UBE1) | P22314 | [64] |
| Ubiquitin-protein ligase EDD1 | UBR5 | (EDD, EDD1, HYD, KIAA0896) | O95071 | [64] |
| U3 snRNA-associated protein 14 A | UTP14A | (SDCCAG16) | Q9BVJ6 | [64] |
| Voltage-dependent anion channel protein 2 | VDAC2 | (VDAC2) | P45880 | [64] |
| Wolframin | WFS1 | (WFS1) | O76024 | [64] |
| X-ray repair cross-complementing protein 5 | XRCC5 | (G22P2, CTC85, CTCBF, Ku80, **Ku86**, TLAA) | P13010 | [64-65] |
| X-ray repair cross-complementing protein 6 | XRCC6 | (G22P1, CTC75, CTCBF, TLAA, **Ku70**) | P12956 | [64-65] |
| Nuclease sensitive element-binding protein 1 | **YBX1** | | Q6PKI6 | [64] |
| **14-3-3 Protein ε** | **YWHAE** | | P62258 | [64] |
| **14-3-3 Protein θ** | **YWHAQ** | | P27348 | [64] |
| **14-3-3 Protein ζ/δ** | **YWHAZ** | | P63104 | [64] |
| Zinc finger CCHC domain-containing protein 3 | ZCCHC3 | | Q3B7J3 | [64] |

**Bold**: Abbreviation used in the text.
Red: Similar or homologous cellular proteins identified in Rep78-associated complexes in both Ad and HSV-1 supported AAV2 replication.

Besides the MCM complex and the processive complex (containing PCNA and DNA polymerase), several other proteins are found in the cellular replisome complex including Claspin, And1, and RFC [84]. A combination of PCNA, POLD1, the helicase complex MCM, and RFC, together with AAV2 Rep have been shown to be required and sufficient to reconstitute efficient AAV2 DNA replication in an *in vitro* replication system [77,78] and all of these proteins were also found in both Ad and HSV-1 supported AAV2 RCs, except for RFC, which was found in AAV2 RCs only in cells coinfected with HSV-1. Rep78 and Rep 68 proteins have been shown to have site-specific DNA helicase and endonuclease activities required to carry out both terminal resolution and reinitiation in an *in vitro* assay [85-87]. Although AAV2 encodes its own helicase provided by Rep, the cellular helicase complex MCM seems to play a central role in AAV2 replication and may not only be involved in formation of the pre-initiation complex. Nash *et al*. suggested that a complex composed of Rep and MCM might bind to the 5′ hairpin after reinitiation of the ITR and unwind the displaced strand from the template, while POLD-PCNA extends the 3′ primer on the template strand [64]. Similar to eukaryotic DNA replication [84], the resulting ss DNA loop of the displaced strand may become coated with the host ss DNA binding protein RPA (composed of the subunits RPA70, RPA32, and RPA14), which is also found in both Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**). However, the observed phosphorylation in infected cells might change the function of RPA32 from DNA replication protein towards DNA damage signaling protein (discussed in more detail below). Premature activation of the DNA replication complex in G1 phase of the cell cycle has been shown to be regulated by binding of retinoblastoma protein (Rb) or prohibitin to MCM proteins

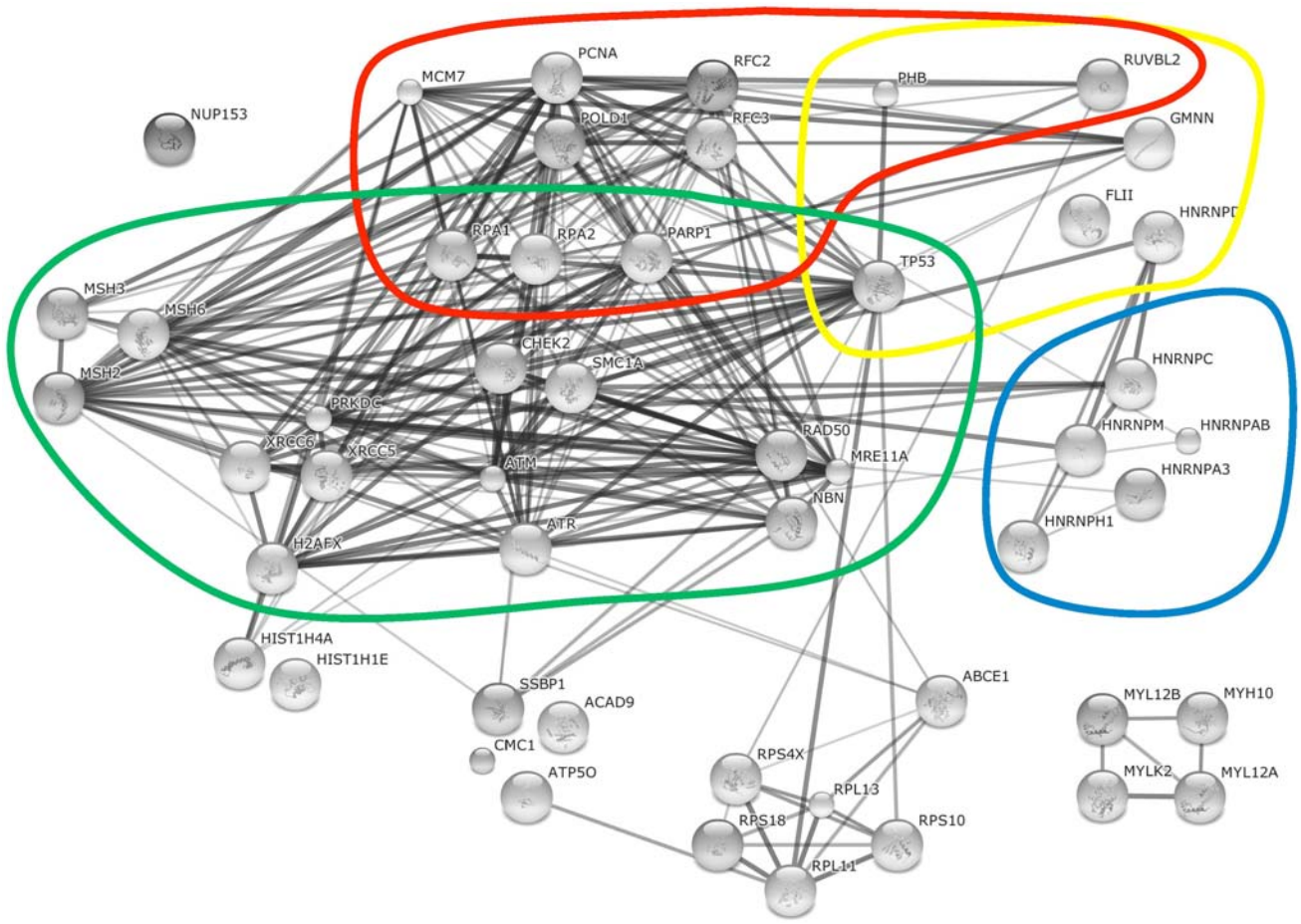



**Fig. (2).** Cellular proteins associated with HSV-1 supported AAV2 RCs. The String database (http://string-db.org/) was used to reconstitute the interaction networks between identified cellular proteins (as described in Figure 1) associated with HSV-1 supported AAV2 RCs and/or the AAV2 Rep78 protein. Four functional categories, mRNA processing (blue), DNA replication (red), transcription (yellow), and DNA damage sensing and repair (green) are highlighted.

[88,89]. Dissociation of prohibitin from MCM is suggested to be regulated by cell cycle regulated kinases and licenses DNA replication in S phase of the cell cycle [89]. Prohibitin (both, PHB and PHB2) was found associated with Rep in both Ad and HSV-1 supported AAV2 replication (Tables **1** and **2**, Fig. **3**; [64,68]). Prohibitin may have an inhibitory effect on regulation of AAV2 DNA replication; however, a direct impact has not yet been investigated. In addition to its role in preventing the activity of replisomes [89], prohibitin might also be involved in transcriptional regulation of viral genes (see below). Cyclin-dependent kinases (CDKs) and germinin are other MCM inhibitors [90]. Binding of both proteins results in the displacement of MCM from replicated DNA at fork termination in late S-phase and prevents re-replication of the cellular genome [90]. In cells coinfected with AAV2 and HSV-1, germinin was found recruited to AAV2 RCs (Table **2**, Fig. **2**; R. Vogel unpublished data). It is possible that a spatial and/or temporal regulation of the activity of the MCM complex by several regulators, including prohibitin and germinin, might also play a central role in the AAV2 life cycle. Spatial or temporal inhibition of AAV2 DNA replication within AAV2 RCs may be important to promote vast transcription of AAV2 genes from ds replication intermediates.

Immunofluorescence analysis revealed a more even distribution of MCM in the nucleus of cells coinfected with AAV2 and HSV-1 although it was also associated with Rep to a certain degree [68]. Similarly, besides significant overlap with Ad supported AAV2 RCs, MCM5 and MCM7 were also detected outside of AAV2 RCs [64]. It is suggested that during HSV-1-induced AAV2 DNA replication Ku proteins may substitute for MCM function in strand displacement activity [68], similar to their partial substitution of MCM in an *in vitro* AAV2 replication assay [64]. In addition, the essential HSV-1 helicase primase complex (UL5/8/52) is proposed to substitute for helicase activity of MCM upon strand displacement [68]. However, dislocation of MCM from AAV2 RCs might also be a consequence of a complex spatial and/or temporal regulation which might influence AAV2 DNA replication in coinfected cells.

### The Role of Cellular DNA Damage Sensing and Repair Proteins in AAV2 DNA Replication

It is likely that the structure of incoming viral DNA as well as viral replication intermediates play a central role in the recruitment of cellular proteins into viral replication compartments. Both incoming Ad and HSV-1 DNA are

**Table 2. Proteins Identified in Rep78-Associated Complexes and/or AAV2 RCs during HSV-1 Supported AAV2 Replication**

| Protein Name | Gene Name (Abbreviation) | Alternative Names | UniProt ID | Reference |
|---|---|---|---|---|
| Serine-protein kinase ATM | **ATM** | (A-T mutated) | Q13315 | [67] |
| Ataxia telangiectasia and Rad3-related protein | **ATR** | (FRP1) | Q13535 | [67] |
| ATP-binding cassette sub-family E member 1 | **ABCE1** | (RLI, RNASEL1, RNASELI, RNS4I, OK/SW-cl.40) | P61221 | [68] |
| Acyl-CoA dehydrogenase family member 9, mitochondrial | **ACAD9** | | Q9H845 | [68] |
| Annexin A5 | ANXA5 | (ANX5, ENX2, PP4) | P08758 | [68] |
| ADP-ribosylation factor-like protein 8B | ARL8B | (ARL10C, GIE1) | Q9NVJ2 | [68] |
| ATP synthase subunit O, mitochondrial | **ATP5O** | (ATPO, OSCP) | P48047 | [68] |
| Serine/threonine-protein kinase Chk2 | CHEK2 | (CDS1, **Chk2**, RAD53) | O96017 | [67] |
| Calcium-binding mitochondrial carrier protein Aralar1 | SLC25A12 | (**CMC1**, ARALAR1) | O75746 | [68] |
| Probable ATP-dependent RNA helicase DDX17 | DDX17 | (DEAD box protein p72, 17, RNA-dependent helicase p72) | Q92841 | [68] |
| Elongation factor 1-alpha 1 | EEF1A1 | (EF1A, LENG7) | P68104 | [68] |
| Protein flightless-1 homolog | **FLII** | (FLIL) | Q13045 | [68] |
| Germinin | GMNN | | O75496 | Vogel unpublished data |
| Histone H2A.x | H2AFX | (**H2AX**, H2a/x) | P16104 | [67] |
| Liver histone H1e | | (H1E) | A3R0T7 | [68] |
| Histone H4 | HIST1H4A | (**H4FA**) | P62805 | [68] |
| Heterogeneous nuclear ribonucleoprotein A3 | HNRNPA3 | (**hnRNPA3**) | P51991 | [68] |
| Heterogeneous nuclear ribonucleoprotein A/B | HNRNPAB | (**hnRNPAB**) | Q53F64 | [68] |
| Heterogeneous nuclear ribonucleoproteins C1/C2 | HNRNPC | (**hnRNPC**) | P07910 | [68] |
| Heterogeneous nuclear ribonucleoprotein D0 | HNRNPD | (AUF1, **hnRNP D**) | Q14103 | [68] |
| Heterogeneous nuclear ribonucleoprotein H | HNRNPH1 | (**hnRPH1**) | P31943 | [68] |
| Heterogeneous nuclear ribonucleoprotein M | HNRNPM | (**hnRPM**, NAGR1) | P52272 | [68] |
| Heat shock cognate 71 kDa protein | HSPA8 | (HSC70, HSP73, HSPA10) | P11142 | [68] |
| Keratin, type I cytoskeletal 13 | KRT13 | | A8K2H9 | [68] |
| Prelamin-A/C (Lamin-A/C) | LMNA | (LMN1) | P02545 | [68] |
| Lamin-B1 | LMNB1 | (LMN2, LMNB) | P20700 | [68] |
| Lamin-B2 | LMNB2 | (LMN2) | Q03252 | [68] |
| LIM domain only protein 7 | LMO7 | (FBX20,FBXO20, KIAA0858) | Q8WWI1 | [68] |
| DNA replication licensing factor MCM7 | **MCM7** | (CDC47) | P33993 | [68] |
| Myosin phosphatase Rho-interacting protein | MPRIP | KIAA0864, MRIP, RHOIP3 | Q6WCQ1 | [68] |
| Double-strand break repair protein MRE11A | MRE11A | (HNGS1, **MRE11**) | P49959 | [68] |
| DNA mismatch repair protein Msh2 | **MSH2** | (MSH2) | P43246 | [68] |
| DNA mismatch repair protein Msh3 | **MSH3** | (DUC1, DUG, MRP1) | P20585 | [68] |
| DNA mismatch repair protein Msh6 | **MSH6** | (GTBP, p160) | P52701 | [68] |
| Myosin-10 | MYH10 | | P35580 | [68] |
| Myosin regulatory light chain 12A | MYL12A | (MLCB, MRLC3, RLC) | P19105 | [68] |
| Myosin regulatory light chain 12B | MYL12B | (MRLC2, MYLC2B) | O14950 | [68] |
| Myosin light chain kinase 2, skeletal/cardiac muscle | MYLK2 | | Q9H1R3 | [68] |

**(Table 2) cond……**

| Protein Name | Gene Name (Abbreviation) | Alternative Names | UniProt ID | Reference |
|---|---|---|---|---|
| Nibrin | NBN | (**NBS1**, P95) | O60934 | [67-68] |
| Nuclear pore complex protein Nup153 | **NUP153** | | P49790 | [68] |
| Poly [ADP-ribose] polymerase 1 | **PARP1** | (ADPRT, PPOL) | P09874 | [68] |
| Proliferating cell nuclear antigen | **PCNA** | | P12004 | [68] |
| Prohibitin | **PHB** | | P35232 | [68] |
| D-3-phosphoglycerate dehydrogenase | PHGDH | (PGDH3) | O43175 | [68] |
| DNA polymerase delta catalytic subunit | **POLD1** | | P28340 | [68] |
| DNA-dependent protein kinase catalytic subunit | PRKDC | (HYRC, HYRC1, DNA-PKcs) | P78527 | [67] |
| DNA repair protein RAD50 | **RAD50** | | Q92878 | [68] |
| RNA-binding protein 14 | RBM14 | (SIP) | Q96PK6 | [68] |
| Replication factor C subunit 2 | **RFC2** | (RFC40) | P35250 | [68] |
| Replication factor C subunit 3 | **RFC3** | | P40938 | [68] |
| Replication protein A 70 kDa DNA-binding subunit | RPA1 | (REPA1, **RPA70**) | P27694 | [68] |
| Replication protein A 32 kDa subunit | RPA2 | (REPA2, **RPA32**, RPA34) | P15927 | [67-68] |
| 60S ribosomal protein L11 | RPL11 | | P62913 | [68] |
| 60S ribosomal protein L13 | RPL13 | (BBC1 OK/SW-cl.46) | P26373 | [68] |
| 40S ribosomal protein S10 | RPS10 | | P46783 | [68] |
| 40S ribosomal protein S18 | RPS18 | | P62269 | [68] |
| RPS4X protein | RPS4X | | P62701 | [68] |
| RuvB-like 2 | **Ruvbl2** | (INO80J, TIP48, TIP49B, CGI-46) | Q9Y230 | [68] |
| Splicing factor, proline- and glutamine-rich | SFPQ | (hPOMp100, SFP) | P23246 | [68] |
| ADP/ATP translocase 3 | SLC25A6 | (ANT3, CDABP0051, ADT3) | P12236 | [68] |
| Structural maintenance of chromosomes protein 1A | SMC1A | (DXS423E, KIAA0178, SB1.8, **SMC1**, SMC1L1) | Q14683 | [68] |
| Spectrin alpha chain, non-erythrocytic 1 | SPTAN1 | (NEAS, SPTA2) | Q13813 | [68] |
| Spectrin beta chain, non-erythrocytic 1 | SPTBN1 | (SPTB2) | Q01082 | [68] |
| Single-stranded DNA-binding protein, mitochondrial | **SSBP1** | (PWP1-interacting protein 17, Mt-SSB) | Q04837 | [68] |
| p53 | TP53 | **(p53)** | P04637 | [67] |
| Tropomodulin-3 | TMOD3 | | Q9NYL9 | [68] |
| Class IVb beta tubulin | | | Q8IWP6 | [68] |
| Elongation factor Tu, mitochondrial | TUFM | (EF-Tu, P43) | P49411 | [68] |
| Vimentin | VIM | | P08670 | [68] |
| X-ray repair cross-complementing protein 5 | XRCC5 | (G22P2, CTC85, CTCBF, Ku80, Ku86, TLAA) | P13010 | [67-68] |
| X-ray repair cross-complementing protein 6 | XRCC6 | (G22P1, CTC75, CTCBF, TLAA, Ku70) | P12956 | [67-68] |

**Bold**: Abbreviation used in the text.
Red: Similar or homologous cellular proteins identified in Rep78-associated complexes in both Ad and HSV-1 supported AAV2 replication.

linear ds molecules that seem to elicit an immediate DDR after entry into the nucleus [91-95]. The incoming AAV2 DNA is of ss nature, with a complex secondary structure. Therefore, it is not surprising that besides RPA several other ssDNA binding proteins including SSBP1, NPM1, RuvBL, and hnRNPU (Tables **1** and **2**, Figs. **1**, **2**) were found associated with AAV2 RCs (discussed in more detail below). In addition, in absence of a helper virus, AAV2 DNA has been shown to elicit a DDR mediated by ATR [82], which is characteristic of the presence of ss DNA ends [96].

There is rising evidence that besides cellular replication factors, also proteins of the DNA sensing and repair machinery play central roles in replication of DNA viruses [91-95]. Three studies have intensively investigated DDR

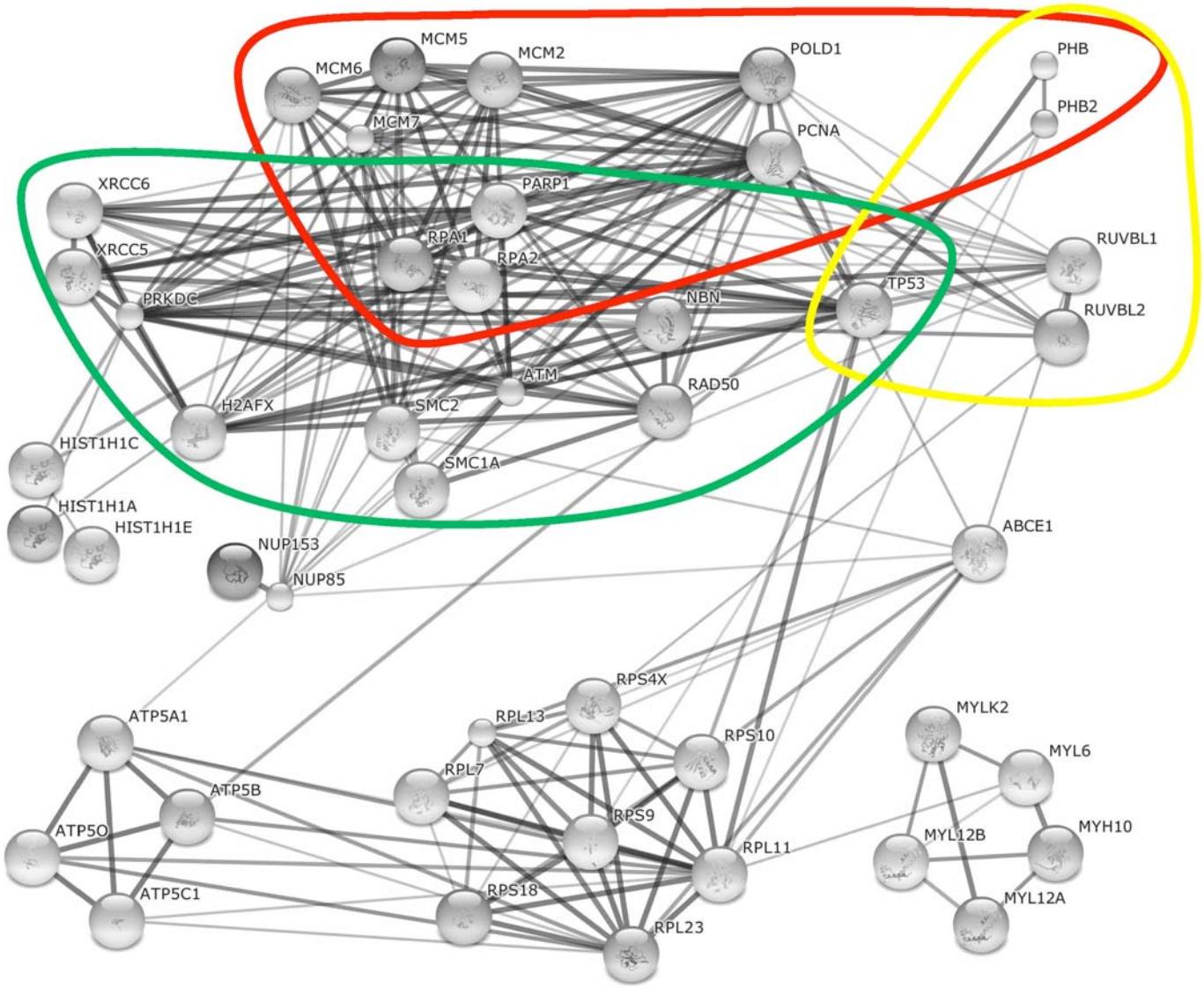



**Fig. (3).** Cellular proteins and homologous proteins found in both Ad and HSV-1 supported AAV2 RCs. The String database (http://string-db.org/) was used to reconstitute the interaction networks between identified cellular proteins or homologous proteins (as described in Fig. **1**) which were found associated with both Ad and HSV-1 supported AAV2 RCs and/or the AAV2 Rep78 protein. Three functional categories, DNA replication (red), transcription (yellow), and DNA damage sensing and repair (green) are highlighted.

factors of Ad or HSV-1 supported AAV2 RCs [65-67]. The main kinase, required for activation of a robust DNA-damage response in AAV2 and Ad coinfected cells has been shown to be DNA-PK [65,66]. In cells coinfected with AAV2 and HSV-1, both ATM and DNA-PK seem to mediate signaling to downstream targets including p53 and Chk2, while signaling to RPA32 appears to be mediated by DNA-PK alone [67]. The DNA-PK complex colocalized with both HSV-1 and Ad supported AAV2 RCs [65,67], however, late upon coinfection with HSV-1, DNA-PKcs was degraded [67]. This might be the reason why DNA-PK was not found associated with AAV2 Rep protein in co-IP experiments [68]. Not only the AAV2 genome structure with its cis-acting replication element CARE, the ITRs, and the single-stranded nature, but also the AAV2 Rep proteins have been shown to evoke a cellular DDR and a cell cycle arrest in S phase [97,98]. The interference of AAV2 with the cellular DDR and repair machinery may not only allow control of cell cycle progression, but also promote second-strand synthesis of the AAV2 genome [74,99]. Cells exposed to a variety of genotoxic agents (e.g. HU or IR) have been shown to support significant helper-independent AAV2 *rep* expression and even DNA replication, although at very low levels [100-103].

Proteins of the DDR and repair machinery also influence rAAV2 vector fate [104-112]. It is suggested that the T-shaped hairpin structure within the AAV ITR and/or ss DNA-ds DNA junctions in the stem of the hairpin, may recruit DNA repair factors of the homologous recombination machinery [113]. While some proteins promote the formation of stable ds rAAV2 genomes, others decrease rAAV2 vector transduction efficiency [104-112]. In addition, genotoxic treatment has been shown to affect also integration rates or rAAV2 genomes; although these events occur at very low frequencies due to the absence of AAV2 *rep* expression [114,115].

Not only in absence of a helper virus, but also upon helper virus supported AAV2 replication, the accumulation of AAV2 replication intermediates harboring a covalently closed hairpin and a free DNA end is very likely to further activate and attract cellular components of the DDR signaling and repair machinery. Therefore, it is conceivably that the composition of cellular proteins in AAV2 RCs may change, depending on the prevalent structure of the AAV2 DNA during infection and replication.

### The Role of the MRN Complex (and its Components) in AAV2 DNA Replication

Proteins of the MRN complex (composed of MRE11, NBS1, and RAD50) are among the first to appear at the site of DNA lesion [116]. MRN mediates downstream signaling to key proteins involved in sensing, signaling, and effector responses to DNA ds breaks [116]. In absence of a helper virus, the MRN complex has been shown to be recruited also to incoming AAV2 DNA [82,104,107]. The consequence of this recruitment on recombinant AAV2 transduction is still under debate.

An inhibitory effect of the MRN complex on transduction efficiency of recombinant AAV2 vectors due to binding of the MRN complex to AAV-ITRs has previously been reported [1,104,106,107,117]. However, upon infection with self-complementary recombinant AAV2 (scAAV) vectors, the MRE11 and NBS1 proteins of the MRN complex have been shown to be required for the formation of double-stranded circular episomes, the predominant form of AAV2 persisting in human tissue [107]. The MRN complex, although with opposite determination, is also a critical element for both helper viruses HSV-1 and Ad [118-125]. The interaction with MRN is one example how Ad and HSV-1 adopt very different ways to interfere with the host DDR. The disruption of the NHEJ machinery by E1b55K/E4orf6 mediated degradation of the MRN complex is absolutely required to prevent Ad genome concatemerization, which would prohibit viral genome packaging into virions [119,126,127]. In addition, Ad interference with these central elements of the DDR and repair system also prevents apoptosis during infection [118,119,128]. In contrast, the MRN complex is required for full activation of the ATM-mediated DDR pathway, which is suggested to support recombination dependent HSV-1 replication and the formation and stabilization of HSV-1 DNA concatemers and complex branched replication products [124,125]. Interestingly, in Ad supported AAV2 replication, the function of MRN for AAV2 replication seems to be linked to its effect on the helper virus. Ad mediated inhibition of the MRN complex has been shown to support AAV2 replication [117], and only the RAD50 and the NBS1 proteins of the MRN complex were detected in Ad supported AAV2 RCs (Table **1**, Fig. **1**). In contrast, all components of the MRN complex (NBS1, MRE11, and RAD50) where found recruited to HSV-1 supported AAV2 RCs (Table **2**, Fig. **2**; [67,68]) and so far, there is no indication of an inhibitory effect of the MRN complex on HSV-1 supported AAV2 replication. It is suggested that the utilization of the HSV-1 polymerase for AAV2 replication may bypass the inhibitory effects of MRN on second-strand synthesis and replication of AAV2 DNA under these helper virus conditions [117]. It remains to be investigated whether the MRN complex may even enhance HSV-1 supported AAV2 replication, as it enhances HSV-1 replication [124,125].

Independent of its function in the MRN complex, phosphorylated NBS1 is involved in the activation of the S-phase checkpoint [129]. In cells coinfected with AAV2 and HSV-1, abundant NBS1 was localized to AAV2 RCs [67], and it may be involved in the induction of a cell cycle arrest in these cells. Although NBS1 levels decrease with the onset of infection [66], a potential role in S-phase checkpoint activation remains to be determined in cells coinfected with AAV2 and Ad as the helpervirus.

In contrast to cells coinfected with AAV2 and Ad, but in line with the observed inhibitory effect of the MRN complex on rAAV2 vector transduction, cells deficient in the MRN target ATM, exhibit increased rAAV2 transduction efficiency [1]. It is suggested that ATM inhibits single-to double-strand conversion of rAAV2 vectors [1].

### The Role of DDR Signaling *via* ATM and DNA-PK in AAV2 DNA Replication

Although the MRN complex is not functional in cells coinfected with AAV2 and Ad, ATM and DNA-PK, two main kinases in DDR [130] mediate downstream signaling to SMC1, Chk1, Chk2, H2AX, XRCC4, and RPA [65,66]. While H2AX, ATM and SMC1 were found in a pan-nuclear pattern, DNA-PK (DNA-PKcs, Ku70, and Ku86) and RPA32 accumulate within Ad supported AAV2 RCs (Table **1**, Fig. **1**; [65,66]). ATM, DNA-PKcs, Chk2, RPA32 and H2AX are phosphorylated and all but H2AX are recruited to HSV-1 supported AAV2 RCs (Table **2**, Fig. **2**; [67,68]). Since all proteins of the MRN complex are also associated with HSV-1 supported AAV2 RCs, it is not clear whether activation of ATM and DNA-PK signaling in these cells requires a functional MRN complex or not. Although the significance of DNA-PK in the AAV2 lytic cycle remains unclear, the activation of the ATM pathway appears to be beneficial for Ad supported AAV2 genome replication [66]. ATM signaling in cells coinfected with AAV2 and Ad may affect SMC1 and SMC2, two central components of condensin and cohesion complexes [131], which were found to be associated with Rep proteins in HSV-1 and Ad supported AAV2 replication (Tables **1** and **2**, Fig. **3**). Both proteins possess DNA ATPase activity and are required for the correct segregation of replicated chromosomes and many other events linked to chromatin dynamics and regulation of gene expression [131]. In addition, the cohesin complex works as a downstream effector in the ATM mediated DDR to control the S-phase checkpoint [132]. It is possible that these factors have also multiple functions in the AAV2 life cycle including checkpoint activation and viral gene expression.

Both ATM and DNA-PK are phosphatidylinositol 3-kinase-related kinases which possess serine/threonine kinase function [133]. 14-3-3 proteins (YWHA) bind to ATM and DNA-PK targets containing phospho-serine or phospho-threonine groups and regulate the function of these proteins implicated for example in cell cycle progression or apoptosis [134]. 14-3-3 proteins ε, θ, and ζ/δ were found associated with Rep proteins in cells coinfected with AAV2 and Ad (Table **1**, Fig. **1**). Although interaction of viral

proteins with 14-3-3 proteins has been described for different viruses [135,136], it has not yet been described for HSV-1 or Ad. It is possible that the interaction of AAV2 Rep with 14-3-3 proteins in cells coinfected with Ad is part of the AAV2 mediated hijacking of cellular factors and might influence cell cycle regulation and/or apoptosis in coinfected cells.

### The Multifunctional RPA Protein and AAV2 Replication

An important downstream target of the DDR is the cellular ss DNA binding protein RPA [137]. Besides its role in DNA replication, RPA is essential also for DNA recombination and repair processes [138]. In this case, the N-terminus of the 32-kDa subunit of human RPA becomes hyperphosphorylated by kinases of the cellular DDR (e.g. DNA-PK; [138]), which is suggested to cause a change in RPA conformation that down-regulates activity in DNA replication but does not affect DNA repair processes [138]. RPA was found in both, Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**) and DNA-PK-dependent phosphorylation of RPA32 has been observed also during both Ad and HSV-1 supported AAV2 replication [65-67]. It is not clear if non-phosphorylated and phosphorylated RPA coexist in AAV2 RCs. But it is possible that temporal phosphorylation of RPA may occur upon AAV2 replication which might influence its function. It is tempting to speculate that in contrast to non-phosphorylated RPA, phosphorylated RPA can participate in DDR signaling upon AAV2 replication and not directly promote the process of AAV2 strand displacement replication *in vivo* [77]. A similar phenomenon has been observed in cells infected with SV40 [139]. Although RPA maintained SV40 DNA replication *in vitro*, it has not been found to be involved in replication of SV40 DNA *in vivo*; more precisely, RPA is phosphorylated *in vivo* and therefore not able to localize to SV40 RCs [139].

### Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR)

While components of the non-homologous end joining (NHEJ) machinery have been shown to have both inhibitory (e.g. Ku70; [140]) and supportive (e.g. DNA ligaseIV/XRCC4; [141]) effects on HSV-1 replication, NHEJ proteins are not beneficial for Ad infection in general [118,119,126-128]. The impact of NHEJ proteins on AAV2 replication is less well understood. Besides the MRN complex and LigaseIV/XRCC4, the central element in NHEJ is the DNA-PK complex [130]. In both, Ad and HSV-1 supported AAV2 replication, DNA-PK was activated and recruited into AAV2 RCs (Tables **1** and **2**, Fig. **3** [65,67]). In addition, signaling to XRCC4 was observed when Ad was the helpervirus [65]. It is suggested that DNA-PK induces the phosphorylation of histone 1 (H1) at DNA ds breaks in order to reduce its affinity for DNA and thereby support access of DNA ligaseIV/XRCC4 to broken DNA ends [142]. Histone 1 (H1F2 or H1E) was also detected associated with both Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**) and may therefore be involved in regulating the access of DNA repair factors to AAV2 DNA. However, another report showed that not the catalytic subunit (cs) of DNA-PK, but Ku70 and Ku80 are required to recruit ligaseIV/XRCC4 to sites of ds breaks and promote end-joining events [143]. It has been shown that Ku proteins directly interact with the AAV2 ITR hairpins [111]. Therefore, both Ku binding to AAV2 DNA [111] as well as the association of DNA-PKcs with Rep78 [64,144] may recruit the DNA-PK complex to AAV2 RCs. However, the role of DNA-PK and other factors of the NHEJ machinery in AAV2 replication is still under debate. In one report, rAAV2 replication in the presence of HSV-1 or Ad helper functions has been shown to be decreased in absence of DNA-PK [111] while another report showed enhanced wtAAV2 genome replication in absence of DNA-PK activity when Ad was used as the helper virus [66]. In the latter case, it has been suggested that loss of DNA-PK could lead to reduced circularization of the AAV2 genome which might promote AAV2 DNA replication events [66]. Indeed, DNA-PK has been shown to support the formation of stable ds rAAV2 vector genomes including circular monomers and concatemers [108,112]. Besides DNA-PKcs, the following repair proteins have been shown to also support rAAV2 recombination events: the MRN complex, ATM, Artemis, BLM, and WRN [105-110,112]. There is evidence that the hairpins at AAV-ITRs are targeted by the cellular DNA repair machinery, including DNA-PK [111] and Artemis [109] similar to DNA hairpin structures in mammalian cells emerging during the V(D)J recombination or NHEJ [145]; this may support viral genome recombination [109], self-circularization [108,112], concatemerization [146], and genomic integration [3] of the rAAV2 genomes. The impact of these proteins on helper virus supported AAV2 replication is not clear. Artemis and ATM are involved in both HR and NHEJ events, depending on the cell-cycle phase [147]. While ATM was found associated with both Ad and HSV-1 supported AAV2 replication (see above, Tables **1** and **2**, Fig. **3**), Artemis as well as BLM and WRN, two proteins of the RecQ helicases complex involved in HR events and stabilization of replicating DNA [148], were not found associated with AAV2 RCs. This is consistent with Ad infection, where BLM is degraded [149], but in contrast to HSV-1 infection, where BML is recruited into viral RCs [140].

Independent of its role in NHEJ events, there is preliminary evidence that DNA-PKcs may mediate also modification of the large Rep proteins upon AAV2 and Ad coinfection [66]. The group of J.P. Trempe showed that phosphorylation of the AAV2 Rep proteins alters their interactions with the AAV2 ITRs [150]; thus DNA-PK may play an essential role in regulating Rep mediated processes of viral DNA replication [65,66], including terminal resolution.

### The Role of NHEJ in AAV2 Integration Events

Although the data is inconsistent, the NHEJ pathway seems to influence AAV2 integration events. In cell culture experiments, DNA-PKcs has been shown to enhance integration of both ss rAAV2 and ds rAAV2 vectors [106]. Another report showed that the NHEJ factor ligase IV in particular supports integration of incoming ss AAV2 vector genomes [151]. Another factor implicated in V(D)J recombination events is the high mobility group protein 1 (HMG1; [152]). Although not identified in presence of Ad and HSV-1, this protein has been shown to bind to Rep78 and enhance RBS binding and nicking activities of Rep as well as site-specific integration of the AAV2 genome in absence of a helper virus [153]. Although these data suggest

a positive influence of NHEJ mechanisms on AAV2 integration in cell culture, rAAV2 genome integration has been shown to be strongly enhanced in DNA-PKcs-deficient SCID mice [154].

**The ATR Pathway**

The MRN complex and BLM as well as several factors of the ATR dependent DDR including ATR, TopBP1, Brca1, Rad17, RPA, Chk1, and Rad51 have been found recruited to AAV2 DNA in absence of a helper virus [82,104]. The group of P. Beard showed that ATR, Chk1, and BML are involved in an AAV2 DNA induced cell cycle arrest in G2 phase, likely by mimicking an aberrant cellular DNA replication fork [82]. Besides the MRN complex, RPA, and ATR, none of the other proteins (BLM, TopBP1, Brca1, Rad17, and Rad51) recruited to AAV2 DNA in absence of a helper virus were found associated with helper virus supported AAV2 RCs. Moreover, for both helper viruses, Ad5 and HSV-1, it has been shown that ATR signaling *via* Chk1 is inhibited in infected cells [67,118,155]. However, factors of the ATR pathway might still have an impact on viral replication, as it has been shown that ATR, RPA, TopBP1, clapsin, and CINP play a beneficial role in HSV-1 gene expression and virus production even in absence of ATR kinase activity [155,156]. The absence and/or obstruction of several proteins of the ATR pathway in presence of a helper virus implies the extensive influence of the helper virus on the cellular environment to promote AAV2 replication.

**PARP**

Another multifunctional cellular repair protein found associated with AAV2 RCs in presence of both Ad and HSV-1 is poly(ADP-ribose) polymerase-1 (PARP-1). This nuclear enzyme mediates poly-ADP-ribosylation (PARylation) of cellular proteins involved in several different processes including replication, recombination, repair, and cell death [157]. The addition of poly-ADP-ribosyl polymers is implicated in the recruitment of DNA damage repair factors to sites of single- and double-strand breaks [157]. In addition, PARP supports DNA stability by protecting DNA ends from nucleases [157]. A recent report showed that upon HSV-1 infection, PARP1/2 is involved in increasing total protein PARylation levels [158], which may occur in the context of DDR activation by HSV-1 [158]. There is one report, showing that the HSV-1 ICP4 protein is PARylated upon infection [159]; however, which effect this modification as well as the overall upregulation of protein PARylation has on HSV-1 infection remains to be investigated. Due to its presence in both Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**), it is very likely that PARP might also affect the function of viral proteins and/or cellular repair and replication factors in AAV2 RCs. In addition, PARP-1 may also be involved in the integration of the AAV2 genome into the host chromosome, similar to its role in HIV integration [160].

**RUVBL1 and RUVBL2**

The RuvB-like proteins (RUVBL1 and RUVBL2), also termed pontin and reptin, are members of the family of ATPases and are associated with diverse cellular activities including regulation of cell proliferation, apoptosis, transcription and DNA repair [161]. Both, pontin and reptin were found associated with Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**). In AAV2 replication, these proteins may be exploited to regulate cell cycle progression or apoptosis. In addition, similar to the AAV2 Rep proteins [75], pontin and reptin both possess ATPase and helicase activity [161] which might also support AAV2 DNA replication.

**NUP85 and NUP153**

The roles of nucleoporins in DNA repair are less well described. Two nucleoporins were found associated with AAV2 RCs, NUP85 and NUP153 (Tables **1** and **2**, Fig. **3**). In yeast, nucleoporins have been shown to be involved in sequestration of active sites of DNA repair to the nuclear periphery [162,163]. In human cells, there is evidence that NUP153 is essential for proper activation of the DNA damage checkpoints and that it promotes NHEJ over HR events [164]. NUP153 may also be involved in supporting NHEJ events in AAV2 infection. In addition, it is tempting to speculate that similar to their role in spatial organization of DNA repair centers in yeast [162,163], nucleoporins may play a role in spatial organization of AAV2 replication compartments.

**Mismatch Repair Proteins**

Another group of DNA repair proteins found in HSV-1 supported AAV2 RCs belong to the family of mismatch repair (MMR) factors. In general, MMR factors are required to maintain DNA integrity [165]. These proteins are highly conserved from prokaryotes to humans and are involved in the recognition of DNA loop or base-base mismatches, resulting from insertion/deletion or DNA polymerase proofreading errors, respectively [165]. Two mismatch repair complexes composed of MSH2 and 3 (MutSβ), and MSH2 and 6 (MutSα) are involved in the recognition of DNA mismatches. MMR factors are also involved in DDR signaling and the control of HR [165]. Similar to HSV-1 infected cells, where MSH2 and 6 have been found associated with the HSV-1 ICP8 protein [140], AAV2 Rep was found associated with MSH2, MSH3, and MSH6 (Table **2**, Fig. **2**; [68]) and the localization of these proteins to HSV-1 supported AAV2 RCs was confirmed by immunofluorescence experiments [68]. It is possible that MSH proteins localize only to HSV-1 but not to Ad supported AAV2 RCs because they bind to the HSV-1 ICP8 protein, which has been shown to both interact with MSH proteins [140] and localize to AAV2 RCs in coinfected cells [68]. Upon HSV-1 infection, MMR proteins have been shown to be not only recruited to viral RCs, but also to be required for efficient HSV-1 replication [156]. It is suggested that besides their proofreading role in viral DNA replication, MMR proteins may play a role in IE gene expression prior to HSV-1 DNA replication [156]. The expression of HSV-1 IE genes is necessary to promote efficient AAV2 replication. In contrast, proteins of the cellular mismatch repair machinery have not been found associated with Ad RCs and do not seem to be involved in efficient virus replication, which may also explain their absence in AAV2 and Ad coinfection. The precise function

of MMR proteins for HSV-1 supported AAV2 replication remains to be elucidated.

### B23/Nucleophosmin

In addition to cellular proteins directly involved in DNA- RNA- or protein-metabolism, some unexpected cellular factors were found associated with AAV2 Rep proteins. For instance B23/nucleophosmin (NPM; Table **1**, Fig. **1**; [64,70]), which is a multifunctional protein involved in duplication of centrosomes [166,167] and protein shuttling [168]. Although it is unclear which of the several functions of NPM may be involved in AAV2 amplification, it has been shown that nucleophosmin participates in Rep-mediated nicking at the AAV2 TRS [70]. Moreover, it is suggested that nucleophosmin has a role also in virion assembly *via* formation of Rep-Cap-NPM complexes [70].

### Transcriptional Regulation

The AAV2 transcripts are generated from three different promoters (p5, p19, and p40) and all pre-mRNAs are generated by the cellular RNA polymerase II complex. Although none of the subunits of the TATA-box dependent transcription initiation complex (formed by TFII, TBP, and Pol II) were identified associated with AAV2 RCs in presence of a helper virus, at least TAF1 (a subunit of TFII; [144]) and TBP [169] were found associated with AAV2 Rep in absence of a helper virus. It is possible that these cellular initiation complex factors are also involved in the initiation of AAV2 transcription, similar to their role in HSV-1 [170] and Ad [171] infection. In addition, it has been shown that the TATA box in cis and the TBP in trans are involved in Rep-dependent replication from the minimal replication origin present within the AAV2 p5 promoter region [172].

Transcriptional activity is not only dependent on the formation of the initiation complex, but is tightly regulated by transcription factors and coactivators [173]. Several transcriptional regulator proteins including Sp1, E2F, p53, and PHB2 as well as transcriptional coactivators including SUB1 and FLII were found in Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Figs. **1**, **2**; [64,72,100,169, 174]). It is worth mentioning that many more proteins involved in transcription (string data base) were found in Ad supported AAV2 RCs (E2F, PHB2, RuvBL1, SSB, SNW1, Sp1, SuB1, SUPT6H, p53, TRIM24, HDAC2, DMAP1, DHX9, YBX1; Table **1**, Fig. **1**) compared to HSV-1 supported AAV2 RCs (PHB, RuvBL2, FLII, hnRNPD, germinin, p53; Table **2**, Fig. **2**). The proteins found in Ad coinfection cover diverse steps of cellular transcription including regulation (E2F or PHB2, string data base), elongation (SUPT6H, string data base), and termination (SSB, string data base), while transcription proteins found in presence of HSV-1 coinfection are mainly regulators of initiation (string data base). Interestingly, in absence of a helper virus, several more cellular transcriptional regulators were found associated with AAV2 Rep including JUN, HMG1, and topors [153,175,176]. It is possible that certain helper virus proteins can substitute for cellular proteins involved in regulation, elongation, and termination of AAV2 transcripts in coinfected cells. For example the HSV-1 ICP4 protein, which is involved in several steps of HSV-1 transcription, including elongation [177], might also be involved in elongation of AAV2 transcripts. For other helper virus proteins, namely the Ad E1A and DBP [52,56,178] as well as the HSV-1 ICP0 protein [57], a positive impact on *rep* gene expression has been shown previously.

### Histones and Histone-Modulating Proteins

As mentioned above, prohibitin is another cellular protein that is recruited into both Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**). Prohibitin is a potential tumor suppressor protein [179]. Similar to Rb, prohibitin mediates suppression of the E2F transcription factor [179]. Prohibitin is recruited into SV40 RCs and represses transcription from SV40 promoters together with histone-deacetylase and N-CoR [180]. Deacetylation of histones leads to a tight binding of histones to the DNA [181] and therefore transcriptional repression.

Upon HSV-1 infection, HDAC2 in a complex with CoREST, LSD1 and REST is involved in the temporal regulation of HSV-1 gene expression by repression of premature transcription of E and L genes [182]. Also upon Ad infection, histone-deacetylase has been shown to interact with E1A in order to regulate temporal expression of the Ad genes [183]. Although not found during AAV2 and HSV-1 coinfection, HDAC2 was found associated with Ad supported AAV2 RCs (Table **1**; Fig. **1**). As already mentioned, E1A has been shown to be required directly for the activation of the AAV2 p5 promoter [56]. Therefore, it is possible that Ad E1A together with prohibitin and histone-deacetylase regulates temporal expression of AAV2 genes *via* modification of histones associated with viral DNA.

Indeed several histone proteins were found in both Ad and HSV-1 supported AAV2 RCs (Tables **1** and **2**, Fig. **3**). This is in line with a report showing that within hours of infection ds AAV2 DNA is associated with nucleosome-like structures, independent of the presence or absence of a helper virus [184]. ds AAV2 DNA associated with nucleosomes may be created either by reassociation of incoming parental ss DNA or by integration into the cellular genome [184]. Similar results have been observed also for autonomous parvoviruses, such as minute virus of mice (MVM; [185]). Histones have also been found to associate with rAAV genomes and induce gene silencing, but expression can be rescued by treatment of the cells with HDAC inhibitor [186].

## POSTTRANSCRIPTIONAL MODIFICATION

### Polyadenylation Capping, and Cap Recognition

In cells pre-mRNA cap formation and polyadenylation are coupled to transcription [187]. Similar to the host RNAs, viral RNAs need to be equipped with 5' Methyl-Gppp caps and 3' poly-adenylated tails or proteins that substitute for these functions, in order to allow nuclear export, translation, and stability of mRNA. All AAV2 transcripts contain a polyadenylation site. Similar to its helper viruses Ad and HSV-1, AAV2 polyadenylation and capping may be performed by host enzymes. Nevertheless, none of the 6 cellular proteins necessary for mRNA polyadenylation (cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors $I_m$ and $II_m$, poly(A) polymerase, and poly(A)-binding protein II (PABP [188]), have been identified as associated with

AAV2 RCs. However, upon HSV-1 infection it has been shown that hnRNPL, a component of the hnRNP complex [189], functions as an adaptor protein to recruit proteins necessary for viral mRNA polyadenylation and nucleocytoplasmic export [190]. Several proteins of the hnRNP complex were indeed found associated with AAV2 RCs when HSV-1 (hnRNPA3, AB, C, D, H1 and M) or Ad (hnRNP U) was the helper virus (Tables **1** and **2**, Figs. **1**, **2**). These components of the hnRNP complex are involved in several functions including polyadenylation, splicing (see below), and localization of mRNAs [189]. Similar to their role in HSV-1 infection, hnRNPs proteins may be involved in polyadenylation and nucleocytoplasmic export of AAV2 mRNAs [190]. In particular hnRNPH, which has been shown to be directly involved in the polyadenylation of cellular mRNAs [189], may support polyadenylation of AAV2 mRNAs in presence of HSV-1.

The eukaryotic initiation factor eIF4F recognizes 5′ caps of mRNAs and mediates initiation of translation *via* recruitment of ribosomes to mRNA [191]. The different functions that can be contributed to its subunits include recognition of the mRNA 5′ cap structure (eIF4E), delivery of an RNA helicase to the 5′ region (eIF4A), bridging of the mRNA and the ribosome (eIF4G), and circularization of the mRNA *via* interaction with PABP [191]. The helicase component eIF4A1 was found associated with Ad supported AAV2 RCs (Table **1**, Fig. **1**).

Even if not found in cells coinfected with AAV2 and HSV-1, eIF4A is also involved in HSV-1 infection, as the HSV-1 endoribonuclease vhs binds to eIF4A to promote degradation of cellular mRNAs [192,193], while HSV-1 RNAs are largely spared from vhs-eIF4A mediated degradation [194]. Which role aIF4A plays for AAV2 replication remains to be investigated and might be dependent on the type of helper virus.

Although not much is known about polyadenylation of AAV2 mRNAs, the presence of AAV2 polyadenylation sites has been shown to be necessary for Rep supported AAV2 pre-mRNA splicing in cells coinfected with Ad [195,196].

### Pre-mRNA Splicing

All AAV2 pre-mRNAs contain introns allowing alternative RNA processing of the overlapping transcription products [195]. Transcription from the p5 and p19 promoters generates mRNAs which encode Rep 78 and Rep 52, respectively; while spliced mRNAs from these promoters encode Rep 68 and Rep 40 [195]. Not only splicing but also alternative splicing plays an important role in the generation of the two different mRNAs generated from the p40 promoter, for translation of the AAV2 capsid proteins VP1 as well as VP2 and VP3 [195]. In addition, the use of two different start codons on the 2.3-kb mRNA, gives rise to VP2 and VP3 [197,198]. As splicing of the nascent AAV2 transcripts is an essential step in AAV2 replication, it is not surprising that one of the main groups of proteins found in Ad and HSV-1 supported AAV2 RCs belong to the cellular splicing machinery including serine/arginine (SR)-rich proteins (SRSFs) and heterogeneous nuclear ribonucleoproteins (hnRNPs; [199]). SRSFs are involved in recruiting the splicing machinery to pre-mRNAs (also called heterogeneous nuclear RNA (hnRNA)), thereby supporting splicing together with hnRNA-binding proteins (hnRNPs; [199]). However, hnRNPs are also implicated in the repression of splicing events by blocking spliceosome assembly [199]. Both SRSF and hnRNP binding sites are located at exon/intron junctions of pre-RNAs [199]. The interplay between SRSF-mediated support and hnRNP-mediated repression of splicing has been found to influence constitutive and alternative splicing events of cellular pre-mRNAs [199]. It is tempting to speculate that similar to their function on cellular pre-mRNAs, SRSFs and/or hnRNPs found in AAV2 RCs (Tables **1** and **2**, Fig. **1** and **2**) regulate constitutive splicing of the two Rep pre-mRNAs from the p5 and p19 promoter [195] as well as alternative splicing of the capsid pre-mRNA from the p40 promoter [195]. Besides the cellular splicing machinery, the AAV2 Rep78/68 proteins as well as helper virus factors are involved in efficient processing of nascent AAV2 transcripts [200]. The Ad factors E2a, E4, and VA in combination with Rep as well as the HSV-1 gene products UL5, UL8, UL52 and UL29 have been shown to stimulate splicing of AAV2 RNA [195]. The AAV2 Rep proteins and helper virus factors might influence the composition and activity of RNA processing factors associated with the RNA polymerase II complex upon AAV2 gene expression; however, the detailed mechanism of Rep and helper virus proteins supported AAV2 mRNA splicing is not yet determined.

Besides their role in mRNA processing, hnRNPs might also be involved in transcriptional regulation, recombination, and telomere maintenance [201]. The reported hnRNP function in protecting single-stranded telomeric DNA repeats from nuclease attacks [201] might also be important for maintaining the integrity of ss AAV2 genomes.

### mRNA EXPORT

mRNA export is intimately coupled to splicing, *via* exon junction complex mediated escort by the export receptor TAP/NXF 1 [202]. However, this factor has not been found associated with AAV2 RCs. Besides TAP/NXF 1, nucleoporins are other central proteins involved in mRNA nuclear export, which were found associated with both Ad (Nup85) and HSV-1 (Nup153) supported AAV2 RCs (Tables **1** and **2**, Fig. **3**). Nucleoporins may contribute to the export of AAV2 mRNAs in coinfected cells. In addition to their function in mRNA export, there is evidence that nucleoporins may also play a role in DNA repair (see above). Two other proteins, hnRNPA3 and RAN, which were found associated with HSV-1 and Ad supported AAV2 RCs, respectively, may also be implicated in viral mRNA trafficking [203].

## CYTOPLASMIC PROTEINS

Several cytoplasmic and mitochondrial proteins were found associated with AAV2 Rep in co-IP experiments in both Ad and HSV-1 supported AAV2 replication [64,68]. The significance of Rep associated with ribosomal factors (RPS and RPL proteins; Tables **1** and **2**, Fig. **3**) is not clear. It is assumed that because of their abundance, ribosomal proteins are often found in proteomic screens [64], and therefore could likely by a contamination of the purified extracts [68]. But associations of Rep with cytoplasmic proteins in general is not entirely unexpected, considering that Rep68 is detected not only in the nuclear [27,45,85], but

also in the cytoplasmic fraction of cells coinfected with AAV2 and Ad [85]. In addition, Rep 78/68 proteins have been previously shown to interact with another cytoplasmic protein, KCTD5, in cells coinfected with AAV2 and Ad [73]. KCTD5 has been shown to act as a substrate-specific adaptor in multimeric cullin E3 ligase reactions, by recruiting proteins for ubiquitination and subsequent proteasome-dependent degradation [204]. It is likely that although only present in small amounts, AAV2 Rep78/68 proteins may have also important functions in the regulation of cytoplasmic proteins such as KCTD5.

Another group of proteins found associated with AAV2 Rep belong to the cellular myosin network (Tables **1** and **2**, Fig. **3**). For HSV-1, interference with cellular myosin has been shown to be implicated in HSV-1 entry processes [205] as well as virion egress [206,207]. In addition, alterations in the distribution of myosin (and actin) filaments in cells infected with HSV-1 have been observed [206-208]. Rep association with these factors might influence the interference of HSV-1 with the cytoskeleton in coinfected cells.

Another cytoplasmic protein associated with Rep78 during both, Ad and HSV-1 supported AAV2 replication is ABCE1 (Tables **1** and **2**, Fig. **3**). In the ATP-binding cassette (ABC) multigene family, the ABCE subfamily is involved in regulation of protein synthesis [209]. Binding of ABCE1 to the eukaryotic initiation factor 2 initiates translation [210]. In addition, ABCE1 interferes with the interferon mediated cellular response against viruses, *via* ribonuclease L (RNase L) of the 2'-5' oligoadenylate/RNase L (2-5A/RNase L) pathway [211]. Several viruses appear to have developed strategies to counteract the antiviral activity of the 2-5A/RNase L pathway including HSV-1 and vaccinia virus [212,213]. It is possible that interaction of Rep 78 with ABCE1 is implicated in counteracting the 2-5A/RNase L activity. On the other hand ABCE1 might support AAV2 capsid assembly, similar to its role in HIV infection, where ABCE1 binds to the HIV-1 Gag and functions as a chaperone by promoting ATP-dependent conformational changes important for HIV capsid assembly and RNA packaging [214].

## MITOCHONDRIAL PROTEINS

As mentioned above, mitochondrial proteins were found associated with AAV2 Rep in co-IP experiments including ATP5B, ATP5C1, HSPA9, ACAD9, ATP5O, SSBP1, and CMC1 (Tables **1** and **2**; Figs. **1**, **2**; [64,68]). It is suggested that the interaction of Rep with several mitochondrial proteins may be a consequence of AAV2 interference with the cells apoptotic pathway [68]. Besides their central role in apoptosis, mitochondria are also central elements of the host defense against viral infections [215]. Therefore, AAV2 interference with mitochondrial proteins may also support productive viral infection by preventing excessive antiviral responses of the host cell.

## CONCLUDING REMARKS

Co-IP experiments as well as immunofluorescence analysis of cells coinfected with AAV2 and helper virus, either Ad or HSV-1, identified numerous cellular proteins that interact with Rep78 and/or are recruited into AAV2 RCs. Clearly additional experiments will be needed to confirm these interactions and to investigate the role of most of these cellular proteins identified in AAV2 RCs in AAV2 replication. In this article, we nevertheless suggest potential functions in fundamental steps of AAV2 replication.

It is conspicuous that irrespective of the type of helper virus (Ad or HSV-1), the largest functional categories of cellular proteins of AAV2 RCs concern DNA replication and repair. In addition several hnRNPs involved in RNA metabolism as well as a minor group of cytoplasmic and mitochondrial proteins were identified as Rep78 interacting partners in presence of either helper virus. By taking a closer look at the composition of the functional categories, it is apparent that the participation of cellular proteins in AAV2 DNA and RNA metabolism depends not only on the specific replication strategy of AAV2, but also on the available helper virus in coinfected cells. Ad and HSV-1 follow very different strategies to replicate their genomes. Ad DNA replication occurs *via* a strand displacement mechanism in which both strands are synthesized in a continuous fashion after protein-primed initiation. In contrast, the basic model for HSV-1 replication is a rolling-circle mechanism including leading- and lagging-stand synthesis. Due to the different modes of replication, it is not surprising that proteins involved in AAV2 replication alter depending on the type of helper virus. Since AAV2 replication occurs by a mechanism distinct from that of both helper viruses, only some of the helper virus replication proteins participate in AAV2 replication, while others have to be substituted by cellular proteins. For example, while Ad supported AAV2 replication uses central cellular components of the DNA replication machinery, such as the MCM complex and polymerase delta, HSV-1 supported AAV2 replication uses helperviral DNA replication proteins including the HSV-1 helicase-primase complex (UL5/UL8/UL52) and the HSV-1 polymerase (UL30; [57-59]). The presence of helper virus substitutes of cellular proteins, and vice versa, may allow AAV2 to expand the host range. Besides cellular and helper virus DNA replication proteins, several factors involved in DNA damage sensing and repair seem to be important for AAV2 replication. It was shown that cells exposed to genotoxic agents (e.g. HU or IR) support efficient helper-independent AAV2 rep expression and low levels of viral DNA replication [100-103]. In addition, for the autonomous parvovirus minute virus of mice, the induction of a DNA damage response facilitates viral replication [216]. Upon coinfection with a helper virus, a defined set of DNA damage sensing and repair proteins were found associated with AAV2 RCs. Some of these proteins, e.g. MMR proteins, were found exclusively in HSV-1 supported AAV2 RCs. MMR proteins may be implicated in proofreading of the viral polymerase and efficient expression of helper virus IE genes [155], which in turn would enhance AAV2 replication. Also, the role of the MRN complex in AAV2 replication seems to depend on its differential effect on the helper virus. Although the effect of many cellular proteins on AAV2 replication is indirect and linked to their effect on the helper virus, other proteins seem to have a more direct role in AAV2 replication. A large number of DNA damage sensing and repair proteins were identified associated with both Ad and HSV-1 supported AAV2 RCs (e.g. RPA, ATM, H2AX, XRCC5/6, and p53). In this context, AAV2 was

shown to modulate the interaction of the helper virus with the cellular DDR [65-67]. Interestingly, while Ad and HSV-1 induce and inactivate different pathways of the DDR to promote their replication [92,94,95], cellular DDR is more similar when coinfected with AAV2. For example, numerous proteins involved in NHEJ are recruited into HSV-1 as well as Ad supported AAV2 RCs [64-68], while in cells infected with Ad alone NHEJ is virtually inactivated. A potential role of NHEJ proteins in AAV2 RCs is to maintain integrity of the viral genome, by protecting the AAV2 DNA from further processing by the AAV2 Rep78 endonuclease activity after nicking at the TRS. In addition, NHEJ components seem to be involved in circularization of incoming AAV2 genomes [108,112], a similar role has been described for retrovirus infection [217]. But not only AAV2 modulates the helper virus induced DDR. The DDR induced by AAV2 alone is completely different from that induced upon coinfection with AAV2 and a helper virus. In that sense, the helper viruses strongly manipulate the AAV2 induced DDR. For example, several proteins of the ATR mediated DDR activated and recruited to AAV2 DNA in absence of a helper virus [82,104] were not found associated with helper virus supported AAV2 RCs. Moreover, in cells coinfected with AAV2 and HSV-1, ATR signaling *via* Chk1 seems to be abrogated [67].

When comparing the minimal set of Ad and HSV-1 helper factors for AAV2 replication, it is conspicuous that several of the helper proteins from Ad but none of the helper proteins provided by HSV-1 are involved in AAV2 RNA metabolism [53,55-59]. Also more cellular proteins involved in RNA metabolism were found associated with Ad supported AAV2 RCs than with HSV-1 supported AAV2 RCs. It is possible that other HSV-1 proteins that do not belong to the minimal set of helper factors can substitute for the cellular RNA metabolism proteins involved AAV2 replication.

As all AAV2 transcripts are spliced [195], it is not surprising that several hnRNPs were found associated with HSV-1 supported AAV2 RCs. Cellular mRNA splicing proteins, including hnRNPs and SRSFs, are even more abundant in Ad supported AAV2 RCs. It would be interesting to define the precise localization of these proteins during AAV2 replication in order to spatially monitor transcriptional activity within AAV2 RCs. For some proteins of the DDR, such a monitoring within/around AAV2 RCs has been performed [65-67] and the data support the idea that AAV2 RCs are well-structured and sub-compartmentalized. In this context, it is suggested that the nuclear matrix acts as a scaffold to which different proteins are recruited and retained [218]. Further studies are needed to determine the importance of such subcompartments for AAV2 replication.

Besides cellular proteins involved in DNA and RNA metabolism, several cytoplasmic and mitochondrial proteins were found associated with AAV2 Rep in co-IP experiments in both Ad and HSV-1 supported AAV2 replication [64,68]. Although the significance of these factors is not known, it suggests that additional cellular pathways not directly involved in DNA and RNA metabolism are targeted by Rep. For example, interaction with cytoplasmic proteins may influence AAV2 virion trafficking while interaction with mitochondrial proteins may interfere with intrinsic responses of the host cell against viral infection [215].

The identification of cellular proteins within helper virus supported AAV2 RCs forms the basis for the investigation of the functional roles of these proteins in AAV2 infection. The identification of cellular inhibitors and enhancers of helper virus supported AAV2 replication will contribute to a better understanding of the complex mechanisms of interaction between AAV2, its helper viruses, and the coinfected cell. Moreover, as AAV2 is a widely used vector in biomedical applications, the detailed knowledge of the functions of cellular proteins in AAV2 gene expression and DNA replication may also help to improve vector production and transduction efficiency.

## SUMMARY

Adeno-associated virus type 2 (AAV2) DNA replication takes place in the host cell nucleus in viral replication compartments (RCs). These compartments have a defined composition of viral and cellular proteins. Co-immunoprecipitation (co-IP) experiments as well as immunofluorescence analysis of cells coinfected with AAV2 and one of its helper viruses, adenovirus (Ad) or herpes simplex virus type 1 (HSV-1), revealed numerous cellular proteins that are recruited into AAV2 RCs or interact with Rep78. The largest functional categories of cellular proteins associated with AAV2 RCs correspond to factors of the DNA replication and repair machinery, independent of the type of helper virus. In addition, proteins involved in RNA metabolism as well as a minor group of cytoplasmic and mitochondrial proteins were identified as Rep78 interacting partners. Although the majority of cellular proteins associated with Ad supported AAV2 RCs was also present in HSV-1 supported AAV2 RCs, some proteins were found associated with AAV2 RCs only in presence of either Ad or HSV-1. The importance of most of the cellular proteins identified in AAV2 RCs for AAV2 replication is not known. Nevertheless, based on the roles of these proteins in cellular processes and in the replication of other viruses, we can make some predictions concerning their functions in different steps of the AAV2 life cycle.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation grants # 31003A_124938 and 31003A_144097.

## REFERENCES

[1] Sanlioglu S, Monick MM, Luleci G, Hunninghake GW, Engelhardt JF. Rate limiting steps of AAV transduction and implications for human gene therapy. Curr Gene Ther 2001; 1: 137-47.

[2] Chejanovsky N, Carter BJ. Mutagenesis of an AUG codon in the adeno-associated virus rep gene: effects on viral DNA replication. Virology 1989; 173: 120-8.

[3] McCarty DM, Young SM, Samulski RJ. Integration of Adeno-Associated Virus (AAV) and Recombinant AAV Vectors. Annu. Rev. Genet. 2004; 38: 819-45.

[4] King JA, Dubielzig R, Grimm D, Kleinschmidt JA. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. EMBO J 2001; 20: 3282-91.

[5] Cassinotti P, Weitz M, Tratschin JD. Organization of the adeno-associated virus (AAV) capsid gene: mapping of a minor spliced

mRNA coding for virus capsid protein 1. Virology 1988; 167: 176-84.

[6] Becerra SP, Koczot F, Fabisch P, Rose JA. Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. J Virol 1988; 62: 2745-54.

[7] Sonntag F, Schmidt K, Kleinschmidt JA. A viral assembly factor promotes AAV2 capsid formation in the nucleolus. Proc Natl Acad Sci USA 2010; 107: 10220-5.

[8] Atchison RW, Casto BC, Hammon WM. Adenovirus-Associated Defective Virus Particles Adenovirus-Associated Defective Virus Particles. Science 1965; 149: 754-6.

[9] Hoggan MD, Blacklow NR, Rowe WP. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proc Natl Acad Sci USA 1966; 55: 1467-74.

[10] Thomson BJ, Weindler FW, Gray D, Schwaab V, Heilbronn R. Human herpesvirus 6 (HHV-6) is a helper virus for adeno-associated virus type 2 (AAV-2) and the AAV-2 rep gene homologue in HHV-6 can mediate AAV-2 DNA replication and regulate gene expression. Virology 1994; 204: 304-11.

[11] Buller RM, Janik JE, Sebring ED, Rose JA. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J Virol 1981; 40: 241-7.

[12] Walz C, Deprez A, Dupressoir T, Durst M, Rabreau M, Schlehofer JR. Interaction of human papillomavirus type 16 and adeno-associated virus type 2 co-infecting human cervical epithelium. J Gen Virol 1997; 78 (Pt 6): 1441-52.

[13] Schnepp BC, Jensen RL, Chen C, Johnson PR, Clark KR. Characterization of Adeno-Associated Virus Genomes Isolated from Human Tissues. J Virol 2005; 79: 14793-803.

[14] Kotin RM, Linden RM, Berns KI. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. EMBO J 1992; 11: 5071-8.

[15] Kotin RM, Menninger JC, Ward DC, Berns KI. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics 1991; 10: 831-4.

[16] Kotin RM, Siniscalco M, Samulski RJ, *et al*. Site-specific integration by adeno-associated virus. Proc Natl Acad Sci USA 1990; 87: 2211-5.

[17] Samulski RJ, Zhu X, Xiao X, *et al*. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J 1991; 10: 3941-50.

[18] Cheung A, Hoggan M, Hauswirth W, Berns K. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J Virol 1980; 33: 739-48.

[19] Im DS, Muzyczka N. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell 1990; 61: 447-57.

[20] Snyder RO, Im DS, Muzyczka N. Evidence for covalent attachment of the adeno-associated virus (AAV) rep protein to the ends of the AAV genome. J Virol 1990; 64: 6204-13.

[21] Hong G, Ward P, Berns KI. *In vitro* replication of adeno-associated virus DNA. Proc Natl Acad Sci USA 1992; 89: 4673-7.

[22] Rekosh DMK, Russell WC, Bellet AJD, Robinson AJ. Identification of a protein linked to the ends of adenovirus DNA. Cell 1977; 11: 83-95.

[23] Roizman B. The checkpoints of viral gene expression in productive and latent infection: the role of the HDAC/CoREST/LSD1/REST repressor complex. J Virol 2011; 85: 7474-82.

[24] Mador N, Panet A, Steiner I. The latency-associated gene of herpes simplex virus type 1 (HSV-1) interferes with superinfection by HSV-1. J Neurovirol 2002; 8: 97-102.

[25] Fraefel C, Bittermann AG, Bueler H, Heid I, Bachi T, Ackermann M. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. J Virol 2004; 78: 389-98.

[26] Heilbronn R, Engstler M, Weger S, Krahn A, Schetter C, Boshart M. ssDNA-dependent colocalization of adeno-associated virus Rep and herpes simplex virus ICP8 in nuclear replication domains. Nucleic Acids Res 2003; 31 :6206-13.

[27] Hunter LA, Samulski RJ. Colocalization of adeno-associated virus Rep and capsid proteins in the nuclei of infected cells. J Virol 1992; 66: 317-24.

[28] Stracker TH, Cassell GD, Ward P, *et al*. The rep protein of adeno-associated virus type 2 interacts with single-stranded DNA-binding proteins that enhance viral replication. J Virol 2004; 78: 441-53.

[29] Weitzman MD, Fisher KJ, Wilson JM. Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. J Virol 1996; 70: 1845-54.

[30] van Bortle K, Corces VG. Nuclear Organization and Genome Function. Annu. Rev. Cell Dev. Biol. 2012; 28: 163-87.

[31] Mao YS, Zhang B, Spector DL. Biogenesis and function of nuclear bodies. Trends in Genetics 2011; 27: 295-306.

[32] Everett RD, Sourvinos G, Leiper C, Clements JB, Orr A. Formation of Nuclear Foci of the Herpes Simplex Virus Type 1 Regulatory Protein ICP4 at Early Times of Infection: Localization, Dynamics, Recruitment of ICP27, and Evidence for the De Novo Induction of ND10-Like Complexes. J Virol 2004; 78: 1903-17.

[33] Everett RD, Sourvinos G, Orr A. Recruitment of herpes simplex virus type 1 transcriptional regulatory protein ICP4 into foci juxtaposed to ND10 in live, infected cells. J Virol 2003; 77: 3680-9.

[34] Maul GG, Ishov AM, Everett RD. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology 1996; 217:67-75.

[35] Lallemand-Breitenbach V, The H de. PML Nuclear Bodies. Cold Spring Harbor Perspectives in Biology 2010; 2:a000661.

[36] Ahn JH, Hayward GS. The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. J Virol 1997; 71: 4599-613.

[37] Doucas V, Ishov AM, Romo A, *et al*. Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. Genes Dev 1996; 10: 196-207.

[38] Leppard KN, Everett RD. The adenovirus type 5 E1b 55K and E4 Orf3 proteins associate in infected cells and affect ND10 components. J Gen Virol 1999; 80 (Pt 4): 997-1008.

[39] Wilkinson GW, Kelly C, Sinclair JH, Rickards C. Disruption of PML-associated nuclear bodies mediated by the human cytomegalovirus major immediate early gene product. J Gen Virol 1998; 79 (Pt 5): 1233-45.

[40] Maul GG, Everett RD. The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. J Gen Virol 1994; 75 (Pt 6): 1223-33.

[41] Bruyn Kops A de, Knipe DM. Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell 1988; 55: 857-68.

[42] Lamberti C, Weller SK. The herpes simplex virus type 1 cleavage/packaging protein, UL32, is involved in efficient localization of capsids to replication compartments. J Virol 1998; 72: 2463-73.

[43] Ward PL, Ogle WO, Roizman B. Assemblons: nuclear structures defined by aggregation of immature capsids and some tegument proteins of herpes simplex virus 1. J Virol 1996; 70: 4623-31.

[44] Hasson TB, Ornelles DA, Shenk T. Adenovirus L1 52- and 55-kilodalton proteins are present within assembling virions and colocalize with nuclear structures distinct from replication centers. J Virol 1992; 66: 6133-42.

[45] Wistuba A, Kern A, Weger S, Grimm D, Kleinschmidt JA. Subcellular compartmentalization of adeno-associated virus type 2 assembly. J Virol 1997; 71: 1341-52.

[46] Yonaha M, Chibazakura T, Kitajima S, Yasukochi Y. Cell cycle-dependent regulation of RNA polymerase II basal transcription activity. Nucleic Acids Res 1995; 23: 4050-4.

[47] Bridge E, Carmo-Fonseca M, Lamond A, Pettersson U. Nuclear organization of splicing small nuclear ribonucleoproteins in adenovirus-infected cells. J Virol 1993; 67: 5792-802.

[48] Pombo A, Ferreira J, Bridge E, Carmo-Fonseca M. Adenovirus replication and transcription sites are spatially separated in the nucleus of infected cells. EMBO J 1994; 13: 5075-5085.

[49] Janik JE, Huston MM, Rose JA. Locations of adenovirus genes required for the replication of adenovirus-associated virus. Proc Natl Acad Sci USA 1981; 78: 1925-9.

[50] Richardson WD, Westphal H. A cascade of adenovirus early functions is required for expression of adeno-associated virus. Cell 1981; 27: 133-41.

[51] Samulski RJ, Shenk T. Adenovirus E1B 55-Mr polypeptide facilitates timely cytoplasmic accumulation of adeno-associated virus mRNAs. J Virol 1988; 62: 206-10.

[52] Tratschin JD, Miller IL, Carter BJ. Genetic analysis of adeno-associated virus: properties of deletion mutants constructed *in vitro* and evidence for an adeno-associated virus replication function. J Virol 1984; 51: 611-9.

[53] Pilder S, Moore M, Logan J, Shenk T. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol Cell Biol 1986; 6: 470-6.

[54] West MH, Trempe JP, Tratschin JD, Carter BJ. Gene expression in adeno-associated virus vectors: the effects of chimeric mRNA structure, helper virus, and adenovirus VA1 RNA. Virology 1987; 160: 38-47.

[55] Carter BJ, Antoni BA, Klessig DF. Adenovirus containing a deletion of the early region 2A gene allows growth of adeno-associated virus with decreased efficiency. Virology 1992; 191: 473-6.

[56] Chang L, Shenk T. The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. J Virol 1990; 64: 2103-9.

[57] Alazard-Dany N, Nicolas A, Ploquin A, *et al*. Definition of herpes simplex virus type 1 helper activities for adeno-associated virus early replication events. PLOS Pathogens 2009; 5: e1000340.

[58] Weindler FW, Heilbronn R. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. J Virol 1991; 65: 2476-83.

[59] Ward P, Falkenberg M, Elias P, Weitzman M, Linden RM. Rep-dependent initiation of adeno-associated virus type 2 DNA replication by a herpes simplex virus type 1 replication complex in a reconstituted system. J Virol 2001; 75: 10250-8.

[60] Challberg MD. A method for identifying the viral genes required for herpesvirus DNA replication. Proc Natl Acad Sci USA 1986; 83: 9094-8.

[61] Slanina H, Weger S, Stow ND, Kuhrs A, Heilbronn R. Role of the herpes simplex virus helicase-primase complex during adeno-associated virus DNA replication. J Virol 2006; 80: 5241-50.

[62] Alex M, Weger S, Mietzsch M, Slanina H, Cathomen T, Heilbronn R. DNA-binding activity of adeno-associated virus rep is required for inverted terminal repeat-dependent complex formation with herpes simplex virus ICP8. J Virol 2012; 86: 2859-63.

[63] Geoffroy M, Salvetti A. Helper functions required for wild type and recombinant adeno-associated virus growth. Curr Gene Ther 2005; 5: 265-71.

[64] Nash K, Chen W, Salganik M, Muzyczka N. Identification of Cellular Proteins That Interact with the Adeno-Associated Virus Rep Protein. J Virol 2008; 83: 454-69.

[65] Schwartz RA, Carson CT, Schuberth C, Weitzman MD. Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. J Virol 2009; 83: 6269-78.

[66] Collaco RF, Bevington JM, Bhrigu V, Kalman-Maltese V, Trempe JP. Adeno-associated virus and adenovirus coinfection induces a cellular DNA damage and repair response *via* redundant phosphatidylinositol 3-like kinase pathways. Virology 2009; 392: 24-33.

[67] Vogel R, Seyffert M, Strasser R, *et al*. Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection. J Virol 2011; 86: 143-55.

[68] Nicolas A, Alazard-Dany N, Biollay C, *et al*. Identification of Rep-Associated Factors in HSV-1-Induced AAV-2 Replication Compartments. J Virol 2012 [Epub ahead of print].

[69] Batchu RB. Dual Level Inhibition of E2F-1 Activity by Adeno-associated Virus Rep78. J Biol Chem 2001; 276: 24315-22.

[70] Bevington JM, Needham PG, Verrill KC, Collaco RF, Basrur V, Trempe JP. Adeno-associated virus interactions with B23/Nucleophosmin: Identification of sub-nucleolar virion regions. Virology 2007; 357: 102-13.

[71] Pereira DJ, Muzyczka N. The cellular transcription factor SP1 and an unknown cellular protein are required to mediate Rep protein activation of the adeno-associated virus p19 promoter. J Virol 1997; 71: 1747-56.

[72] Batchu RB, Shammas MA, Wang JY, Munshi NC. Interaction of adeno-associated virus Rep78 with p53: implications in growth inhibition. Cancer Res 1999; 59: 3592-5.

[73] Weger S, Hammer E, Gotz A, Heilbronn R. Identification of a cytoplasmic interaction partner of the large regulatory proteins Rep78/Rep68 of adeno-associated virus type 2 (AAV-2). Virology 2007; 362: 192-206.

[74] Ferrari FK, Samulski T, Shenk T, Samulski RJ. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J Virol 1996; 70: 3227-34.

[75] Weitzman MD, Linden RM. In: Snyder RO, Moullier P, editors. Adeno-Associated Virus. Totowa, NJ: Humana Press; 2011, p. 1-23.

[76] Diffley JFX. Regulation of early events in chromosome replication. Curr Biol 2004; 14: R778-86.

[77] Nash K, Chen W, Muzyczka N. Complete in vitro reconstitution of Adeno-associated virus DNA replication requires the minichromosome maintenance complex proteins. J Virol 2008; 82: 1159-64.

[78] Ni TH, McDonald WF, Zolotukhin I, *et al*. Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. J Virol 1998; 72: 2777-87.

[79] Myers MW, Laughlin CA, Jay FT, Carter BJ. Adenovirus helper function for growth of adeno-associated virus: effect of temperature-sensitive mutations in adenovirus early gene region 2. J Virol 1980; 35: 65-75.

[80] Lee SS, Lehman IR. Unwinding of the box I element of a herpes simplex virus type 1 origin by a complex of the viral origin binding protein, single-strand DNA binding protein, and single-stranded DNA. Proc Natl Acad Sci USA 1997; 94: 2838-42.

[81] Prindle MJ, Loeb LA. DNA polymerase delta in dna replication and genome maintenance. Environ Mol Mutagen 2012; 53: 666-82.

[82] Jurvansuu J, Raj K, Stasiak A, Beard P. Viral Transport of DNA Damage That Mimics a Stalled Replication Fork. J Virol 2004; 79: 569-80.

[83] Waga S, Bauer G, Stillman B. Reconstitution of complete SV40 DNA replication with purified replication factors. J Biol Chem 1994; 269: 10923-34.

[84] Leman A, Noguchi E. The Replication Fork: Understanding the Eukaryotic Replication Machinery and the Challenges to Genome Duplication. Genes 2013; 4: 1-32.

[85] Im DS, Muzyczka N. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell 1990; 61: 447-57.

[86] Snyder RO, Im DS, Muzyczka N. Evidence for covalent attachment of the adeno-associated virus (AAV) rep protein to the ends of the AAV genome. J Virol 1990; 64: 6204-13.

[87] Zhou X, Zolotukhin I, Im DS, Muzyczka N. Biochemical characterization of adeno-associated virus rep68 DNA helicase and ATPase activities. J Virol 1999; 73: 1580-90.

[88] Sterner JM, Dew-Knight S, Musahl C, Kornbluth S, Horowitz JM. Negative regulation of DNA replication by the retinoblastoma protein is mediated by its association with MCM7. Mol Cell Biol 1998; 18: 2748-57.

[89] Rizwani W, Alexandrow M, Chellappan S. Prohibitin physically interacts with MCM proteins and inhibits mammalian DNA replication. Cell Cycle 2009; 8: 1621-9.

[90] Blow JJ, Dutta A. Preventing re-replication of chromosomal DNA. Nat Rev Mol Cell Biol 2005; 6: 476-86.

[91] Jiang M, Imperiale MJ. Design stars: how small DNA viruses remodel the host nucleus. Future Virology 2012; 7: 445-59.

[92] Turnell AS, Grand RJ. DNA viruses and the cellular DNA-damage response. J Gen Virol 2012; 93: 2076-97.

[93] Nikitin PA, Luftig MA. At a crossroads: human DNA tumor viruses and the host DNA damage response. Future Virology 2011; 6: 813-30.

[94] Lilley CE, Schwartz RA, Weitzman MD. Using or abusing: viruses and the cellular DNA damage response. Trends Microbiol 2007; 15: 119-26.

[95] Weitzman MD, Carson CT, Schwartz RA, Lilley CE. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) 2004; 3: 1165-73.

[96] Shiotani B, Zou L, Eds. A Human Cell Extract-Based Assay for the Activation of ATM and ATR Checkpoint Kinases. New York, NY.: Humana Press, c/o Springer Science+Business Media 2011.

[97] Berthet C, Raj K, Saudan P, Beard P. How adeno-associated virus Rep78 protein arrests cells completely in S phase. Proc Natl Acad Sci USA 2005; 102: 13634-9.

[98] Saudan P, Vlach J, Beard P. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. EMBO J 2000; 19: 4351-61.

[99] Fisher KJ, Gao GP, Weitzman MD, DeMatteo R, Burda JF, Wilson JM. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. J Virol 1996; 70: 520-32.

[100] Nicolas A, Jolinon N, Alazard-Dany N, *et al*. Factors influencing helper-independent adeno-associated virus replication. Virology 2012; 432: 1-9.

[101] Yalkinoglu AO, Heilbronn R, Burkle A, Schlehofer JR, Zur Hausen H. DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. Cancer Res 1988; 48: 3123-9.

[102] Yakobson B, Hrynko TA, Peak MJ, Winocour E. Replication of adeno-associated virus in cells irradiated with UV light at 254 nm. J Virol 1989; 63: 1023-30.

[103] Yakobson B, Koch T, Winocour E. Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. J Virol 1987; 61: 972-81.

[104] Cervelli T, Palacios JA, Zentilin L, *et al*. Processing of recombinant AAV genomes occurs in specific nuclear structures that overlap with foci of DNA-damage-response proteins. J Cell Sci 2008; 121: 349-57.

[105] Sanlioglu S, Benson P, Engelhardt JF. Loss of ATM function enhances recombinant adeno-associated virus transduction and integration through pathways similar to UV irradiation. Virology 2000; 268: 68-78.

[106] Cataldi MP, McCarty DM. Differential effects of DNA double-strand break repair pathways on single-strand and self-complementary adeno-associated virus vector genomes. J Virol 2010; 84: 8673-82.

[107] Choi VW, McCarty DM, Samulski RJ. Host cell DNA repair pathways in adeno-associated viral genome processing. J Virol 2006; 80: 10346-56.

[108] Duan D, Yue Y, Engelhardt JF. Consequences of DNA-dependent protein kinase catalytic subunit deficiency on recombinant adeno-associated virus genome circularization and heterodimerization in muscle tissue. J Virol 2003; 77: 4751-9.

[109] Inagaki K, Ma C, Storm TA, Kay MA, Nakai H. The role of DNA-PKcs and artemis in opening viral DNA hairpin termini in various tissues in mice. J Virol 2007; 81: 11304-21.

[110] Nakai H, Storm TA, Fuess S, Kay MA. Pathways of removal of free DNA vector ends in normal and DNA-PKcs-deficient SCID mouse hepatocytes transduced with rAAV vectors. Hum Gene Ther 2003; 14: 871-81.

[111] Choi YK, Nash K, Byrne BJ, Muzyczka N, Song S. The effect of DNA-dependent protein kinase on adeno-associated virus replication. PloS ONE 2010; 5: e15073.

[112] Song S, Laipis PJ, Berns KI, Flotte TR. Effect of DNA-dependent protein kinase on the molecular fate of the rAAV2 genome in skeletal muscle. Proc Natl Acad Sci USA 2001; 98: 4084-8.

[113] Adachi K, Nakai H (eds.). The Role of DNA Repair Pathways in Adeno-Associated Virus Infection and Viral Genome Replication / Recombination / Integration. New York, NY: InTech 2011.

[114] Russell DW, Alexander IE, Miller AD. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. Proc Natl Acad Sci USA 1995; 92: 5719-23.

[115] Fragkos M, Breuleux M, Clement N, Beard P. Recombinant Adeno-Associated Viral Vectors Are Deficient in Provoking a DNA Damage Response. J Virol 2008; 82: 7379-87.

[116] Williams GJ, Lees-Miller SP, Tainer JA. Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. DNA Repair (Amst) 2010; 9: 1299-306.

[117] Schwartz RA, Palacios JA, Cassell GD, Adam S, Giacca M, Weitzman MD. The Mre11/Rad50/Nbs1 complex limits adeno-associated virus transduction and replication. J Virol 2007; 81: 12936-45.

[118] Carson C, Schwartz R, Stracker T, Lilley C, Lee D, Weitzman M. The Mre11 complex is required for ATM activation and the G2/M checkpoint. EMBO J 2003; 22: 6610-20.

[119] Stracker TH, Carson CT, Weitzman MD. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature 2002; 418: 348-52.

[120] Karen KA, Hearing P. Adenovirus core protein VII protects the viral genome from a DNA damage response at early times after infection. J Virol 2011; 85: 4135-42.

[121] Sohn S, Hearing P. Adenovirus regulates sumoylation of Mre11-Rad50-Nbs1 components through a paralog-specific mechanism. J Virol 2012; 86: 9656-65.

[122] Araujo FD, Stracker TH, Carson CT, Lee DV, Weitzman MD. Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes. J Virol 2005; 79: 11382-91.

[123] Liu Y, Shevchenko A, Shevchenko A, Berk AJ. Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. J Virol 2005; 79: 14004-16.

[124] Lilley CE, Carson CT, Muotri AR, Gage FH, Weitzman MD. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci USA 2005; 102: 5844-9.

[125] Wilkinson DE, Weller SK. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J Virol 2004; 78: 4783-96.

[126] Baker A, Rohleder KJ, Hanakahi LA, Ketner G. Adenovirus E4 34k and E1b 55k Oncoproteins Target Host DNA Ligase IV for Proteasomal Degradation. J Virol 2007; 81: 7034-40.

[127] Boyer J, Rohleder K, Ketner G. Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. Virology 1999; 263: 307-12.

[128] Querido E, Blanchette P, Yan Q, *et al*. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs *via* a novel mechanism involving a Cullin-containing complex. Genes Dev 2001; 15: 3104-17.

[129] Lavin MF, Kozlov S. ATM activation and DNA damage response. Cell Cycle 2007; 6: 931-42.

[130] Giglia-Mari G, Zotter A, Vermeulen W. DNA damage response. Cold Spring Harbor perspectives in biology. Genes Dev 2011; 3: a000745.

[131] Legagneux V, Cubizolles F, Watrin E. Multiple roles of Condensins: a complex story. Biol Cell 2004; 96: 201-13.

[132] Yazdi PT, Wang Y, Zhao S, Patel N, Lee EYP, Qin J. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. Genes Dev 2002;16:571-82.

[133] Abraham RT. PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. DNA Repair 2004; 3: 883-7.

[134] Gardino AK, Yaffe MB. 14-3-3 proteins as signaling integration points for cell cycle control and apoptosis. Semin Cell Dev Biol 2011; 22: 688-95.

[135] Pei Z, Harrison MS, Schmitt AP. Parainfluenza virus 5 m protein interaction with host protein 14-3-3 negatively affects virus particle formation. J Virol 2011; 85: 2050-9.

[136] Aoki H, Hayashi J, Moriyama M, Arakawa Y, Hino O. Hepatitis C virus core protein interacts with 14-3-3 protein and activates the kinase Raf-1. J Virol 2000; 74: 1736-41.

[137] Wang H, Guan J, Perrault AR, Wang Y, Iliakis G. Replication protein A2 phosphorylation after DNA damage by the coordinated action of ataxia telangiectasia-mutated and DNA-dependent protein kinase. Cancer Res 2001; 61: 8554-63.

[138] Binz SK, Sheehan AM, Wold MS. Replication protein A phosphorylation and the cellular response to DNA damage. DNA Repair 2004; 3: 1015-24.

[139] Vassin VM, Wold MS, Borowiec JA. Replication protein A (RPA) phosphorylation prevents RPA association with replication centers. Mol Cell Biol 2004; 24: 1930-43.

[140] Taylor TJ, Knipe DM. Proteomics of herpes simplex virus replication compartments. J Virol 2004; 78: 5856-66.

[141] Muylaert I, Elias P. Knockdown of DNA Ligase IV/XRCC4 by RNA Interference Inhibits Herpes Simplex Virus Type I DNA Replication. J Biol Chem 2007; 282: 10865-72.

[142] Kysela B, Chovanec M, Jeggo PA. Phosphorylation of linker histones by DNA-dependent protein kinase is required for DNA ligase IV-dependent ligation in the presence of histone H1. Proc Natl Acad Sci USA 2005; 102: 1877-82.

[143] Mari P, Florea BI, Persengiev SP, *et al*. Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. Proc Natl Acad Sci USA 2006; 103: 18597-602.

[144] Pegoraro G, Marcello A, Myers MP, Giacca M. Regulation of adeno-associated virus DNA replication by the cellular TAF-I/set complex. J Virol 2006;80:6855-64.

[145] Le Deist F, Poinsignon C, Moshous D, Fischer A, Villartay J de. Artemis sheds new light on V(D)J recombination. Immunol Rev 2004; 200: 142-55.

[146] Nakai H, Thomas CE, Storm TA, *et al*. A limited number of transducible hepatocytes restricts a wide-range linear vector dose response in recombinant adeno-associated virus-mediated liver transduction. J Virol 2002; 76: 11343-9.

[147] Goodarzi AA, Jeggo P, Lobrich M. The influence of heterochromatin on DNA double strand break repair: Getting the strong, silent type to relax. DNA Repair 2010; 9: 1273-82.

[148] Chu WK, Hickson ID. RecQ helicases: multifunctional genome caretakers. Nat Rev Cancer 2009; 9: 644-54.

[149] Orazio NI, Naeger CM, Karlseder J, Weitzman MD. The adenovirus E1b55K/E4orf6 complex induces degradation of the Bloom helicase during infection. J Virol 2011; 85: 1887-92.

[150] Narasimhan D, Collaco R, Kalman-Maltese V, Trempe JP. Hyper-phosphorylation of the adeno-associated virus Rep78 protein inhibits terminal repeat binding and helicase activity. Biochim Biophys Acta 2002; 1576: 298-305.

[151] Daya S, Cortez N, Berns KI. Adeno-associated virus site-specific integration is mediated by proteins of the nonhomologous end-joining pathway. J Virol 2009; 83: 11655-64.

[152] Lieber MR, Wilson TE. SnapShot: Nonhomologous DNA end joining (NHEJ). Cell 2010; 142: 496-496.e1.

[153] Costello E, Saudan P, Winocour E, Pizer L, Beard P. High mobility group chromosomal protein 1 binds to the adeno-associated virus replication protein (Rep) and promotes Rep-mediated site-specific cleavage of DNA, ATPase activity and transcriptional repression. EMBO J 1997; 16: 5943-54.

[154] Song S, Lu Y, Choi YK, *et al*. DNA-dependent PK inhibits adeno-associated virus DNA integration. Proc Natl Acad Sci USA 2004; 101: 2112-6.

[155] Mohni KN, Dee AR, Smith S, Schumacher AJ, Weller SK. Efficient Herpes Simplex Virus 1 Replication Requires Cellular ATR Pathway Proteins. J Virol 2012; 87: 531-42.

[156] Mohni KN, Mastrocola AS, Bai P, Weller SK, Heinen CD. DNA Mismatch Repair Proteins Are Required for Efficient Herpes Simplex Virus 1 Replication. J Virol 2011; 85: 12241-53.

[157] Thomas C, Tulin AV. Poly-ADP-ribose polymerase: Machinery for nuclear processes. Mol Aspects Med 2013; doi: pii: S0098-2997 (13)00025-3.

[158] Grady SL, Hwang J, Vastag L, Rabinowitz JD, Shenk T. Herpes simplex virus 1 infection activates poly(ADP-ribose) polymerase and triggers the degradation of poly(ADP-ribose) glycohydrolase. J Virol 2012; 86: 8259-68.

[159] Blaho JA, Michael N, Kang V, *et al*. Differences in the poly(ADP-ribosyl)ation patterns of ICP4, the herpes simplex virus major regulatory protein, in infected cells and in isolated nuclei. J Virol 1992; 66: 6398-407.

[160] Ha HC. Poly(ADP-ribose) polymerase-1 is required for efficient HIV-1 integration. Proc Natl Acad Sci USA 2001; 98: 3364-8.

[161] Huber O, Menard L, Haurie V, Nicou A, Taras D, Rosenbaum J. Pontin and reptin, two related ATPases with multiple roles in cancer. Cancer Res 2008; 68: 6873-6.

[162] Nagai S, Dubrana K, Tsai-Pflugfelder M, *et al*. Functional targeting of DNA damage to a nuclear pore-associated SUMO-dependent ubiquitin ligase. Science 2008; 322: 597-602.

[163] Oza P, Jaspersen SL, Miele A, Dekker J, Peterson CL. Mechanisms that regulate localization of a DNA double-strand break to the nuclear periphery. Genes Dev 2009; 23: 912-27.

[164] Lemaitre C, Fischer B, Kalousi A, *et al*. The nucleoporin 153, a novel factor in double-strand break repair and DNA damage response. Oncogene 2012; 31: 4803-9.

[165] Jiricny J. MutLalpha: at the cutting edge of mismatch repair. Cell 2006; 126: 239-41.

[166] Okuda M. The role of nucleophosmin in centrosome duplication. Oncogene 2002; 21: 6170-4.

[167] Okuda M, Horn HF, Tarapore P, *et al*. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell 2000; 103: 127-40.

[168] Szebeni A, Herrera JE, Olson MO. Interaction of nucleolar protein B23 with peptides related to nuclear localization signals. Biochemistry 1995; 34: 8037-42.

[169] Hermonat PLea. The adeno-associated virus Rep78 major regulatory protein binds the cellular TATA-binding protein *in vitro* and *in vivo*. Virology 1998; 245: 120-7.

[170] Grondin B, DeLuca N. Herpes simplex virus type 1 ICP4 promotes transcription preinitiation complex formation by enhancing the binding of TFIID to DNA. J Virol 2000; 74: 11504-10.

[171] Kawase H, Okuwaki M, Miyaji M, *et al*. NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. Genes Cells 1996; 1: 1045-56.

[172] Francois A, Guilbaud M, Awedikian R, Chadeuf G, Moullier P, Salvetti A. The cellular TATA binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 p5 element. J Virol 2005; 79: 11082-94.

[173] Maston GA, Evans SK, Green MR. Transcriptional regulatory elements in the human genome. Annu Rev Genomics Hum Genet 2006; 7: 29-59.

[174] Pereira D.J., McCarty DM Muzyczka N. The adeno-associated virus (AAV) Rep protein acts as both a repressor and an activator to regulate AAV transcription during a productive infection. J Virol 1997; 71: 1079-88.

[175] Prasad C, Meyers C, Zhan D, *et al*. The adeno-associated virus major regulatory protein Rep78-c-Jun-DNA motif complex modulates AP-1 activity. Virology 2003; 314: 423-31.

[176] Weger S, Hammer E, Heilbronn R. Topors, a p53 and topoisomerase I binding protein, interacts with the adeno-associated virus (AAV-2) Rep78/68 proteins and enhances AAV-2 gene expression. J Gen Virol 2002; 83: 511-6.

[177] Wagner LM, Lester JT, Sivrich FL, DeLuca NA. The N terminus and C terminus of herpes simplex virus 1 ICP4 cooperate to activate viral gene expression. J Virol 2012; 86: 6862-74.

[178] Laughlin CA, Jones N, Carter BJ. Effect of deletions in adenovirus early region 1 genes upon replication of adeno-associated virus. J Virol 1982; 41: 868-76.

[179] Wang S, Nath N, Adlam M, Chellappan S. Prohibitin, a potential tumor suppressor, interacts with RB and regulates E2F function. Oncogene 1999; 18: 3501-10.

[180] Wang S, Fusaro G, Padmanabhan J, Chellappan SP. Prohibitin co-localizes with Rb in the nucleus and recruits N-CoR and HDAC1 for transcriptional repression. Oncogene 2002; 21: 8388-96.

[181] Hayakawa T, Nakayama J. Physiological roles of class I HDAC complex and histone demethylase. J Biomed Biotechnol 2011; 2011: 129383.

[182] Roizman B, Zhou G, Du T. Checkpoints in productive and latent infections with herpes simplex virus 1: conceptualization of the issues. J Neurovirol 2011; 17: 512-7.

[183] Gallimore PH, Turnell AS. Adenovirus E1A: remodelling the host cell, a life or death experience. Oncogene 2001; 20: 7824-35.

[184] Marcus-Sekura CJ, Carter BJ. Chromatin-like structure of adeno-associated virus DNA in infected cells. J Virol 1983; 48: 79-87.

[185] Ben-Asher E, Bratosin S, Aloni Y. Intracellular DNA of the parvovirus minute virus of mice is organized in a minichromosome structure. J Virol 1982; 41: 1044-54.

[186] Okada T, Uchibori R, Iwata-Okada M, *et al*. A histone deacetylase inhibitor enhances recombinant adeno-associated virus-mediated gene expression in tumor cells. Mol Ther 2006; 13: 738-46.

[187] Yonaha M, Proudfoot NJ. Transcriptional termination and coupled polyadenylation *in vitro*. EMBO J 2000; 19: 3770-7.

[188] Zhao J, Hyman L, Moore C. Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. Microbiol Mol Boil 1999; 63: 405-45.

[189] Chaudhury A, Chander P, Howe PH. Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: Focus on hnRNP E1's multifunctional regulatory roles. RNA 2010; 16: 1449-62.

[190] Guang S, Felthauser AM, Mertz JE. Binding of hnRNP L to the pre-mRNA processing enhancer of the herpes simplex virus thymidine kinase gene enhances both polyadenylation and nucleocytoplasmic export of intronless mRNAs. Mol Cell Biol 2005; 25: 6303-13.

[191] Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 1999; 68: 913-63.

[192] Feng P, Everly DN, Read GS. mRNA Decay during Herpesvirus Infections: Interaction between a Putative Viral Nuclease and a Cellular Translation Factor. J Virol 2001; 75: 10272-80.

[193] Feng P, Everly DN, Read GS. mRNA Decay during Herpes Simplex Virus (HSV) Infections: Protein-Protein Interactions Involving the HSV Virion Host Shutoff Protein and Translation Factors eIF4H and eIF4A. J Virol 2005; 79: 9651-64.

[194] Taddeo B, Zhang W, Roizman B. The herpes simplex virus host shutoff RNase degrades cellular and viral mRNAs made before infection but not viral mRNA made after infection. J Virol 2013; 87: 4516-22.

[195] Qiu J., Pintel D. Processing of adeno-associated virus RNA. Front Biosci. 2008; 1: 3101-15.

[196] Mouw MB, Pintel DJ. Adeno-associated virus RNAs appear in a temporal order and their splicing is stimulated during coinfection with adenovirus. J Virol 2000; 74: 9878-88.

[197] Becerra SP, Rose JA, Hardy M, Baroudy BM, Anderson CW. Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon. Proc Natl Acad Sci USA 1985; 82: 7919-23.

[198] Muralidhar S, Becerra SP, Rose JA. Site-directed mutagenesis of adeno-associated virus type 2 structural protein initiation codons: effects on regulation of synthesis and biological activity. J Virol 1994; 68: 170-6.

[199] Busch A, Hertel KJ. Evolution of SR protein and hnRNP splicing regulatory factors. Wiley Interdiscip Rev RNA 2012; 3: 1-12.

[200] Qiu J, Pintel DJ. The adeno-associated virus type 2 Rep protein regulates RNA processing *via* interaction with the transcription template. Mol Cell Biol 2002; 22: 3639-52.

[201] Krecic AM, Swanson MS. hnRNP complexes: composition, structure, and function. Curr Opin Cell Biol 1999; 11: 363-71.

[202] Reed R, Magni K. A new view of mRNA export: separating the wheat from the chaff. Nat Cell Biol 2001; 3: E201-4.

[203] Ma ASW, Moran-Jones K, Shan J, *et al.* Heterogeneous nuclear ribonucleoprotein A3, a novel RNA trafficking response element-binding protein. J Biol Chem 2002; 277: 18010-20.

[204] Bayon Y, Trinidad AG, La Puerta ML de, *et al*. KCTD5, a putative substrate adaptor for cullin3 ubiquitin ligases. FEBS J 2008; 275: 3900-10.

[205] Arii J, Goto H, Suenaga T, *et al.* Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1. Nature 2010; 467: 859-62.

[206] van Leeuwen H, Elliott G, O'Hare P. Evidence of a role for nonmuscle myosin II in herpes simplex virus type 1 egress. J Virol 2002; 76: 3471-81.

[207] Roberts KL, Baines JD. Myosin Va enhances secretion of herpes simplex virus 1 virions and cell surface expression of viral glycoproteins. J Virol 2010; 84: 9889-96.

[208] Winkler M, Dawson GJ, Elizan TS, Berl S. Distribution of actin and myosin in a rat neuronal cell line infected with herpes simplex virus. Arch Virol 1982; 72: 95-103.

[209] Tyzack JK, Wang X, Belsham GJ, Proud CG. ABC50 interacts with eukaryotic initiation factor 2 and associates with the ribosome in an ATP-dependent manner. J Biol Chem 2000; 275: 34131-9.

[210] Chen Z, Dong J, Ishimura A, Daar I, Hinnebusch AG, Dean M. The essential vertebrate ABCE1 protein interacts with eukaryotic initiation factors. J Biol Chem 2006; 281: 7452-7.

[211] Bisbal C, Silhol M, Laubenthal H, *et al.* The 2'-5' oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. Mol Cell Biol 2000; 20: 4959-69.

[212] Cayley PJ, Davies JA, McCullagh KG, Kerr IM. Activation of the ppp(A2'p)nA system in interferon-treated, herpes simplex virus-infected cells and evidence for novel inhibitors of the ppp(A2'p)nA-dependent RNase. Eur J Biochem 1984; 143: 165-74.

[213] Paez E, Esteban M. Resistance of vaccinia virus to interferon is related to an interference phenomenon between the virus and the interferon system. Virology 1984; 134: 12-28.

[214] Lingappa JR, Dooher JE, Newman MA, Kiser PK, Klein KC. Basic residues in the nucleocapsid domain of Gag are required for interaction of HIV-1 gag with ABCE1 (HP68), a cellular protein important for HIV-1 capsid assembly. J Biol Chem 2006; 281: 3773-84.

[215] Ohta A, Nishiyama Y. Mitochondria and viruses. Mitochondrion 2011; 11: 1-12.

[216] Adeyemi RO, Landry S, Davis ME, Weitzman MD, Pintel DJ. Parvovirus minute virus of mice induces a DNA damage response that facilitates viral replication. PLOS Pathogens 2010; 6: e1001141.

[217] Li L, Olvera JM, Yoder KE, *et al.* Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. EMBO J 2001; 20: 3272-81.

[218] Jong RN de, van der Vliet PC, Brenkman AB. Adenovirus DNA replication: protein priming, jumping back and the role of the DNA binding protein DBP. Curr Top Microbiol Immunol 2003; 272: 187-211.

Received: August 12, 2013 Revised: September 26, 2013 Accepted: September 30, 2013

# 4 Results

## 4.1 A differential analysis of DNA damage signaling in cells infected with HSV-1 or coinfected with AAV2 and HSV-1.

Numerous studies revealed that viruses interfere with the cellular DNA damage sensing and repair machinery (17, 23, 26, 35, 36) and proteins of the DDR pathway have been shown to significantly influence viral replication (17, 23, 26, 35, 36). I have performed a detailed analysis of the DDR induced in cells infected with HSV-1 alone or coinfected with HSV-1 and AAV2, and the results are shown here in the form of a manuscript that has been published in the Journal of Virology.

*My contribution to this publication was as follows: design of the experiments, main experimental work, including co-immunoprecipitation, Western and immunofluorescence analyses, confocal microscopy, flow cytometry, and writing of manuscript. The co-authors contributed to this work by providing reagents, and some technical and editorial help.*

Journal of Virology

# Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection

**Rebecca Vogel, Michael Seyffert, Regina Strasser, Anna P. de Oliveira, Christiane Dresch, Daniel L. Glauser, Nelly Jolinon, Anna Salvetti, Matthew D. Weitzman, Mathias Ackermann and Cornel Fraefel**

***J. Virol.*** **2012, 86(1):143. DOI: 10.1128/JVI.05694-11.**
**Published Ahead of Print 19 October 2011.**

JVI
Journals.ASM.org

# Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection

**Rebecca Vogel,[a] Michael Seyffert,[a] Regina Strasser,[a] Anna P. de Oliveira,[a] Christiane Dresch,[a] Daniel L. Glauser,[b] Nelly Jolinon,[c] Anna Salvetti,[c] Matthew D. Weitzman,[d]* Mathias Ackermann,[a] and Cornel Fraefel[a]**

Institute of Virology, University of Zurich, Zurich, Switzerland[a]; Division of Virology, Department of Pathology, University of Cambridge, Cambridge, United Kingdom[b]; INSERM U758, Ecole Normale Supérieure de Lyon, Lyon, France[c]; and The Salk Institute for Biological Studies, La Jolla, California, USA[d]

**Adeno-associated virus type 2 (AAV2) is a human parvovirus that relies on a helper virus for efficient replication. Herpes simplex virus 1 (HSV-1) supplies helper functions and changes the environment of the cell to promote AAV2 replication. In this study, we examined the accumulation of cellular replication and repair proteins at viral replication compartments (RCs) and the influence of replicating AAV2 on HSV-1-induced DNA damage responses (DDR). We observed that the ATM kinase was activated in cells coinfected with AAV2 and HSV-1. We also found that phosphorylated ATR kinase and its cofactor ATR-interacting protein were recruited into AAV2 RCs, but ATR signaling was not activated. DNA-PKcs, another main kinase in the DDR, was degraded during HSV-1 infection in an ICP0-dependent manner, and this degradation was markedly delayed during AAV2 coinfection. Furthermore, we detected phosphorylation of DNA-PKcs during AAV2 but not HSV-1 replication. The AAV2-mediated delay in DNA-PKcs degradation affected signaling through downstream substrates. Overall, our results demonstrate that coinfection with HSV-1 and AAV2 provokes a cellular DDR which is distinct from that induced by HSV-1 alone.**

Adeno-associated virus type 2 (AAV2) is a small, nonenveloped parvovirus with a single-stranded DNA genome of 4.7 kb (52). In the absence of a helper virus, AAV2 establishes a latent infection characterized by site-specific integration of the viral genome into the AAVS1 site on human chromosome 19 (72). In the presence of a helper virus, AAV2 can replicate productively in the host cell nucleus. AAV2 DNA replication occurs at discrete sites in the nucleus, termed replication compartments (RCs). During the course of infection, several small RCs rapidly expand and fuse to large structures, which displace the cellular chromatin and fill the entire cell nucleus (28, 35, 37, 79, 91). AAV2 RCs contain AAV2 proteins, as well as defined helper virus proteins and cellular proteins (3, 35, 63, 65, 75, 79, 90, 91). Replicating AAV2 has inhibitory effects on both the host cell (9, 41, 68, 71, 73, 74, 100, 101) and the helper virus (5, 30, 31, 34, 40, 44, 61, 84, 100).

One of the helper viruses for AAV2 replication is herpes simplex virus 1 (HSV-1) (14). The minimal HSV-1 helper factors for AAV2 replication from plasmid substrates include the helicase-primase complex encoded by UL5, UL8, and UL52 and the major DNA binding protein ICP8 (3) (90). Besides viral helper factors, the fate of AAV2 replication also depends on cellular proteins. Recently, cellular proteins have been identified that interact with AAV2 Rep78/68 in adenovirus (Ad)- or HSV-1-supported AAV2 replication (63, 65). Of these, the largest functional categories correspond to cellular proteins which are involved in DNA metabolism, including DNA replication, repair, and chromatin modification.

There is accumulating evidence that the DNA damage response (DDR) pathways play central roles in viral replication (92). Control of DDR signaling may be a mechanism to prevent apoptosis and/or stop cell cycle progression (92). For example, DNA damage signaling has been shown to enhance the replication of the autonomous parvovirus minute virus of mice, perhaps in part by promoting cell cycle arrest (1). In response to DNA damage, a complex signaling network is activated that includes kinase regulation, transcriptional induction, and redistribution of a multitude of factors (33, 38). Depending on the extent of DNA damage, cell cycle progression is stopped to repair DNA breaks or apoptosis is induced. Two main pathways are classified for the repair of DNA double-strand breaks, homologous recombination and nonhomologous end joining (16, 36, 99). Proteins which are important for sensing of DNA double-strand breaks include H2AX and the Mre11/Rad50/Nbs1 (MRN) complex (for a review, see reference 47). The phosphatidylinositol-3-kinase-like kinases (PIKKs) ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR) are proximal signaling kinases that have key functions in signaling transduction in homologous recombination (24, 33, 60, 66, 69). ATM is recruited by the MRN complex (for a review, see reference 29) and catalytically activated through dimer dissociation and autophosphorylation at serine 1981 (S1981) (6, 103). Examination of ATR recruitment to sites of DNA damage revealed that binding of ATR to ATR-interacting protein (ATRIP) leads to colocalization of the ATR-ATRIP complex with replication protein A (RPA)-coated single-stranded DNA (7). It has been suggested that interaction of topoisomerase II-binding protein 1 with the ATR-ATRIP complex induces kinase activity of ATR (59). A third PIKK, DNA-dependent protein kinase (DNA-PK), belongs to the nonhomologous end-joining machinery and is composed of the Ku70/Ku80 heterodimer and the catalytic subunit of DNA-PK (DNA-PKcs). Ku70/80 directly recognizes DNA double-strand breaks and activates DNA-PKcs (for a review, see reference 15). Activity of DNA-PKcs is proposed to be regulated

**Received** 17 July 2011 **Accepted** 10 October 2011
**Published ahead of print** 19 October 2011
Address correspondence to Cornel Fraefel, cornel.fraefel@access.uzh.ch.
* Present address: Matthew D. Weitzman, Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA.
Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.05694-11

by autophosphorylation at several sites, including S2056 (19, 21). Investigation of downstream signaling via PIKKs suggests that checkpoint kinase 1 (Chk1) is mainly a substrate of ATR after the recognition of single-strand breaks and stalled-replication forks (22, 32, 53, 80, 83, 105), while Chk2 activation by ATM is more restricted to double-strand breaks, including those induced by ionizing radiation (2, 20, 42, 43, 56, 57). However, there is evidence that ATR (85, 87) and DNA-PK (50, 85) can also induce Chk2 phosphorylation. DNA-PK (49, 86, 88), ATR (46), and ATM (8) have all been reported to induce phosphorylation of p53.

HSV-1 induces the activation of a cellular DNA double-strand break response pathway involving the MRN complex, ATM, p53, RPA (nonphosphorylated), and Rad51 (13, 51, 76, 96), while the ATR response has been reported to be inhibited (58, 94). Similarly, signaling via DNA-PK is also inhibited by HSV-1 through ICP0-dependent proteasomal degradation of DNA-PKcs (48, 67). It has been shown that in the absence of the Ku70 subunit of the DNA-PK complex, HSV-1 replication is enhanced (81).

AAV2, although not replicating in the absence of a helper virus, induces a strong DDR mediated by ATR (41). A different DDR is induced upon coinfection with a helper virus that supports AAV2 replication; for example, during Ad-supported AAV2 replication, the DDR signaling is mediated primarily by DNA-PK and is independent of the MRN complex (25, 75). Furthermore, activation of ATM, Chk1, Chk2, RPA, and H2AX was also observed (25, 75). Given that DNA-PK is a key kinase in nonhomologous end joining, it seems that these events may play important roles not only in the (site-specific) integration of the AAV2 genome (18, 27, 77) but also in AAV2 genome replication (23). As opposed to Ad and AAV2 coinfection, the DDR induced by coinfection with HSV-1 and AAV2 has not previously been investigated. Thus, the goals of the present study were to identify cellular replication and repair proteins that accumulate at AAV2 RCs when HSV-1 is the helper virus and to determine the effect of AAV2 on the cellular DDR signaling pathways induced by HSV-1.

## MATERIALS AND METHODS

**Cells.** DNA-PKcs-positive (expressing one copy of DNA-PKcs) and DNA-PKcs-negative HCT116 cells were kindly provided by E. Hendrickson (University of Minnesota Medical School, Minneapolis) and maintained in growth medium containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 $\mu$g/ml streptomycin, and 0.25 $\mu$g/ml amphotericin B (1% AB). DNA-PKcs-positive (Fus1) and DNA-PKcs-negative (Fus9) MO59J cells were kindly provided by T. Melendy (Department of Cellular and Molecular Biology, Roswell Park Cancer Institute, Buffalo, NY) and cultured in 50% F10 medium–50% DMEM supplemented with 10% FBS, 1% AB, and 250 $\mu$g/ml G418. AT22 IJE-T yZ5 (expressing ATM) and AT22 IJE-T pEBS7 (lacking ATM) fibroblast cells were kind gifts from Y. Shiloh (Department of Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). These cells were maintained in DMEM supplemented with 10% FBS, 1% AB, and 100 $\mu$g/ml hygromycin B. U2OS GW33 cells were a kind gift from P. Nghiem (Department of Medicine/Dermatology, University of Washington, Seattle) and were cultured in DMEM containing 10% FBS, 1% AB, 200 $\mu$g/ml G418, and 50 $\mu$g/ml hygromycin B. Vero cells were maintained in DMEM supplemented with 10% FBS and 1% AB. All cells were maintained at 37°C in a 95% air–5% $CO_2$ atmosphere.

**Viruses.** HSV-1 strain F was kindly provided by B. Roizman (Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, Chicago, IL). rHSV-1 dl1403 (rHSV-1ΔICP0) was kindly provided by N. D. Stow (MRC Virology Unit, University of Glasgow, Glasgow, United Kingdom), and rHSV-1vEYFP-ICP4 and rHSV-1 vECFP-ICP4 were a kind gift R. D. Everett (MRC Virology Unit, University of Glasgow, Glasgow, United Kingdom). Viruses were grown on Vero cells. HSV-1 strain F titers were determined on Vero cells, and rHSV-1 dl1403 titers were determined on U2OS cells. AAV2 and Ad2 were kindly provided by H. Buening (University of Cologne, Cologne, Germany) and U. Greber (University of Zurich, Zurich, Switzerland), respectively. The recombinant AAV2CherryRep (rAAVCR) genome, containing the AAV2 inverted terminal repeats flanking the *rep* open reading frames fused at its 5′ terminus with the mCherry coding sequence, has been described previously (3). rAAVCR particles of AAV serotype 2 were produced by transient transfection of 2935Z with pDG and pAAVCR and purified on two successive CsCl gradients, and titers of genome-containing particles were determined by dot blot assay. rAAV2GFP was kindly provided by M. Linden (King's College London School of Medicine, London, United Kingdom).

**Antibodies.** The following primary antibodies were used: anti-actin (Santa Cruz Biotechnology SC-10731; dilution for Western blotting [WB], 1:10,000), anti-ATM (Genetex 70107; dilution for WB, 1:1,000), anti-ATM-P-S1981 (Rockland Immunochemicals 200-301-400; dilution for WB, 1:500; dilution for immunofluorescence [IF] assay, 1:50), anti-ATR (SC-28901; dilution for WB, 1:1,000), anti-ATR-P-S428 (Cell Signaling Technology CST-2853; dilution for WB, 1:500; dilution for IF assay, 1:200), anti-ATRIP (Abcam Ab-19531; dilution for WB, 1:2,000; dilution for IF assay, 1:500), anti-Chk1 (SC-8408; dilution for WB, 1:1,000), anti-Chk1-P-S345 (CST-133D3; dilution for WB, 1:1,000), anti-Chk2 (SC-5278; dilution for WB, 1:1,000), anti-Chk2-P-T68 (SC-16297; dilution for WB and immunoprecipitation, 1:250; dilution for IF assay, 1:100), anti-DNA-PKcs (NeoMarkers M5370; dilution for WB, 1:500; dilution for IF assay, 1:50; dilution for flow cytometry, 1:250), anti-DNA-PKcs-P-S2056 (Ab-18192; dilution for WB, 1:2,000; dilution for IF assay, 1:500), anti-HSV-1 ICP8 (Ab-20193; dilution for WB, 1:1,000; dilution for IF assay, 1:200), anti-H2AX-P-S139 (Millipore 05-636; dilution for WB, 1:500; dilution for IF assay, 1:50), anti-Nbs1 (Novus Biologicals 100-143; dilution for WB, 1:1,000), anti-Nbs1-P-S343 (Ab-47272; dilution for WB, 1:500; dilution for IF assay, 1:100), anti-p53 (Ab-1101; dilution for WB, 1:1,000), anti-p53-P-S15 (Ab-38497 [purchased in June 2009 and no longer available]; dilution for WB, 1:500; dilution for IF assay, 1:500), anti-AAV2 Rep (Fitzgerald Industries 10R-A111A; dilution for WB, 1:200), anti-RPA32 (Bethyl Laboratories BL-A300-244A; dilution for WB, 1:2,000; dilution for IF assay, 1:500), anti-RPA32-P-S4/8 (BL-A300-245A; dilution for WB, 1:200; dilution for IF assay, 1:200), and anti-USP7 (CST-3277; dilution for WB, 1:750). The following secondary antibodies were used: rabbit anti-mouse IgG-horseradish peroxidase (HRP; Sigma A9044; dilution, 1:10,000), goat anti-rabbit IgG-HRP (Sigma A6154; dilution, 1:10,000), rabbit TrueBlot, goat anti-rabbit IgG (H+L)-Alexa Fluor 405 (AF405; Molecular Probes A31556; dilution, 1:500), goat anti-rabbit IgG (H+L)-AF594 (Molecular Probes A11012; dilution, 1:1,000), goat anti-mouse IgG (H+L)-AF594 (Molecular Probes A11005; dilution, 1:1,000), goat anti-mouse IgG (H+L)-fluorescein isothiocyanate (FITC; Southern Biotechnology 1031-02; dilution, 1:200), goat anti-rabbit IgG (H+L)-FITC (Southern Biotechnology 4050-02; dilution, 1:200), and goat anti-mouse IgG-Cy5 (Millipore AP181S; dilution, 1:500).

**WB analysis.** A total of $10^6$ HCT116 cells, $5 \times 10^6$ AT22 IJE-T, or $5 \times 10^6$ MO59J fusion cells were seeded into 6-cm plates. The following day, the cells were mock infected, infected with either AAV2 (multiplicity of infection [MOI], 2,000) or HSV-1 (MOI, 1.5), or coinfected with AAV2 (MOI, 2,000) and HSV-1 (MOI, 1.5) in DMEM supplemented with 2% FBS and 1% AB. Cells treated for 18 h with hydroxyurea (HU; 3 mM) served as a positive control for activation of DDR proteins. After 3 h, 6 h, 9 h, 12 h, 24 h, or 48 h, cells were trypsinized, washed once with phosphate-buffered saline (PBS), and resuspended in 200 $\mu$l EBC-170 lysis buffer (50 mM Tris-HCl, 170 mM NaCl, 0.5% Nonidet P-40, Complete Mini-EDTA-free protease inhibitor [Roche Diagnostics, Rotkreuz, Switzerland]). After 30 min of incubation at 4°C under constant agitation, the suspension was centrifuged (20 min at 13,200 $\times$ *g*) and the superna-

tant was collected, mixed with 2× loading buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.005% bromphenol blue, 0.125 M Tris-HCl, pH 6.8), and boiled for 10 min. Cell lysates were separated, depending on the molecular weight of the protein of interest, on 8%, 10%, or 12% SDS-polyacrylamide gels and transferred to Protran nitrocellulose membranes (Whatman, Bottmingen, Switzerland). For detection of γH2AX, proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane with a pore size of 0.45 μm (Amersham Hybond-P; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The membranes were blocked with PBS-T (PBS containing 0.3% Tween 20) supplemented with 5% nonfat dry milk for 1 h at room temperature (RT). Incubation with antibodies was carried out with PBS-T supplemented with 2.5% nonfat dry milk. Primary antibodies were incubated overnight at 4°C, while secondary antibodies were incubated for 1 h at RT. Membranes were washed three times with PBS-T for 10 min after each antibody incubation step. HRP-conjugated secondary antibodies were detected with ECL detection reagent (ECL WB blotting systems; GE Healthcare, Zurich, Switzerland). The membranes were exposed to chemiluminescence detection films (Roche Diagnostics, Rotkreuz, Switzerland). Detection of anti-actin served as a loading control for the lysate.

**Fluorescence-activated cell sorting (FACS) and WB analysis.** A total of $6.6 \times 10^6$ AT22 IJE-T cells were seeded into 10-cm cell culture dishes. Cells were mock infected, infected with rHSV-1vECFP-ICP4 (MOI, 2 or 4), or coinfected with rHSV-1vECFP-ICP4 (MOI, 2 or 4) and rAAV2CR (MOI, 4,000). Cells positive for mCherry (rAAV2) or ICP4-ECFP (HSV-1) were sorted and prepared for WB analysis as described above. The same number of mock-infected and HU-treated cells (3 mM, 18 h postinfection [hpi]) was used as a control.

**Immunoprecipitation.** AT22 IJE-T cells (90,000) were seeded into 6-well plates. The next day, cells were mock infected, infected with either AAV2 (MOI, 2,000) or HSV-1 (MOI, 1.5), or coinfected with AAV2 (MOI, 2,000) and HSV-1 (MOI, 1.5) in DMEM supplemented with 2% FBS and 1% AB. After 24 h, cells were trypsinized, washed once with PBS, and resuspended in 200 μl EBC-170 lysis buffer containing the primary antibody. After incubation for 2 h at 4°C under constant agitation, 30 μl protein A Sepharose CL-4B (GE Healthcare, Zurich, Switzerland) was added and the mixture was incubated for 2 h at 4°C under constant agitation. Then, Sepharose beads were pelleted and the supernatant was collected as loading and infection controls. The pellet was washed twice with PBS for 15 min at 4°C under constant agitation, and protein was eluted using 20 to 40 μl of a 4 M urea solution (pH 7.5). After 10 min of incubation at 4°C under constant agitation, beads were pelleted and the supernatant was collected. Samples were analyzed by WB assay as described above; however, for detection of immunoprecipitated proteins, the secondary antibody rabbit IgG TrueBlot (anti-rabbit IgG-HRP; eBioscience, San Diego, CA) was used, which preferentially detects the nonreduced form of rabbit IgG over the reduced, SDS-denatured form of IgG.

**IF analysis.** HCT116 cells ($5 \times 10^4$), AT22 IJE-T cells ($7{,}5 \times 10^4$), or MO59J fusion cells ($7{,}5 \times 10^4$) were seeded onto coverslips (12-mm diameter; Glaswarenfabrik Karl Hecht GmbH & Co. KG, Sondheim, Germany) in 24-well plates. The next day, cells were mock infected, infected with HSV-1 (MOI, 1.5), or coinfected with rAAV2CR (MOI, 250) and HSV-1 (MOI, 1.5), rHSV-1dl1403 (MOI, 0.9), or Ad2 (MOI, 12). UV-exposed cells (10 J/m$^2$) or HU (3 mM)-treated cells served as a positive control for activation of DDR proteins. After 24 h, cells were washed once with cold PBS and fixed with 3.7% formaldehyde in PBS for 15 min at RT. The fixation process was stopped by incubation with 0.1 M glycine for 10 min at RT. Cells were washed twice with cold PBS. For permeabilization, cells were treated for 2 min with precooled (−20°C) acetone and washed three times with PBS. Cells were blocked for 30 min with 3% bovine serum albumin (BSA) in PBS. For staining, cells were incubated with antibodies diluted in PBS-BSA (3%) in a humidified chamber at RT in the dark. Coverslips were placed on droplets (40 μl) of primary antibody solution. After incubation for 1 h, cells were washed three times with PBS. DAPI (4′,6′-diamidino-2-phenylindole) staining was performed together with the secondary antibody staining. For this, DAPI (0.5 μg/ml) and the secondary antibody were diluted in PBS-BSA (3%). After incubation for 1 h at RT in the dark, cells were washed three times with PBS and once with $H_2O$. Coverslips were embedded in Glycergel (DakoCytomation, Carpinteria, CA) containing DABCO (26 mg/ml; Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany), and cells were observed using a confocal laser scanning microscope (CLSM; Leica TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany). To prevent cross talk between the channels for the different fluorochromes, all channels were recorded separately and fluorochromes with longer wavelengths were recorded first. Images from the CLSM were deconvolved with Huygens Essential 2.6.0p1 software (Scientific Volume Imaging, Hilversum, Netherlands) and processed using Imaris 5.0.1 (Bitplane AG, Zurich, Switzerland).

**Flow cytometry.** The day before infection, $10^6$ HCT116 cells were seeded into 6-cm tissue culture plates. The cells were mock infected; infected with rHSV-1vEYFP-ICP4 (MOI, 1.5); or coinfected with AAV2 (MOI, 250), rAAV2GFP (MOI, 250), and HSV-1 (MOI, 1.5) in DMEM supplemented with 2% FCS and 1% AB; and incubated for 20 h at 37°C in a 5% $CO_2$ atmosphere. Cells were trypsinized and washed once with PBS. For fixation and staining, the BD Cytofix/Cytoperm Plus kit (BD Biosciences) was used according to the manufacturer's instructions. Flow cytometry was performed on a FACScalibur (BD). DNA-PKcs contents were analyzed in HSV-1-infected cells positive for EYFP-ICP4 (rHSV-1 encoded) and in coinfected cells positive for enhanced green fluorescent protein (EGFP; rAAV encoded). A minimum of 80,000 events were scored for each sample. The mean fluorescence intensity of cell populations was determined using FlowJo software (FlowJo version 8.6.3; Stanford University, Stanford, CA).

## RESULTS

**Activation of primary DNA damage markers Nbs1 and H2AX upon virus infection.** We compared the DDRs of cells infected with HSV-1 alone or coinfected with AAV2. As we have previously observed that at low MOIs, HSV-1-supported AAV2 RCs are not detectable before approximately 18 hpi, we analyzed the cells for up to 48 hpi.

The overall levels of Nbs1 were not altered by virus infection (Fig. 1A). However, Nbs1 was phosphorylated upon infection with HSV-1 alone or coinfection with AAV2 (Fig. 1B); moreover, phosphorylated Nbs1-P-S343 colocalized with AAV2 RCs and with HSV-1 RCs (Fig. 2A). Nbs1 expression was also examined in AT22 IJE-T and HCT116 cells with similar results (data not shown). In HCT116 cells, HSV-1 infection- and coinfection-induced phosphorylation was also indicated by shifted Nbs1 bands (data not shown). In addition, H2AX was found to be phosphorylated (Fig. 1A) and to surround HSV-1-supported AAV2 RCs (Fig. 2B). As previously observed, H2AX also surrounded HSV-1 RCs (Fig. 2B) (94).

**Coinfection with AAV2 and HSV-1 induces the phosphorylation of ATM and ATR.** Previous studies revealed that ATM-mediated signaling is induced (51, 76, 96) and that ATR-mediated signaling is blocked in HSV-1-infected cells (58, 94). To examine the activity of these two PIKKs in cells coinfected with HSV-1 and AAV2, we first determined their phosphorylation status by WB and IF analyses. While total ATM levels were similar in virus-infected cells and mock-infected cells (Fig. 1A), ATM-dependent staining with an antibody generated to autophosphorylated ATM was detected only in cells infected with HSV-1 alone or coinfected with AAV2 (Fig. 1B) and in these cells it colocalized with HSV-1 and AAV2 RCs (Fig. 3A). As this antibody detects other phosphorylated targets of ATM such as 53BP1, this finding only demon-

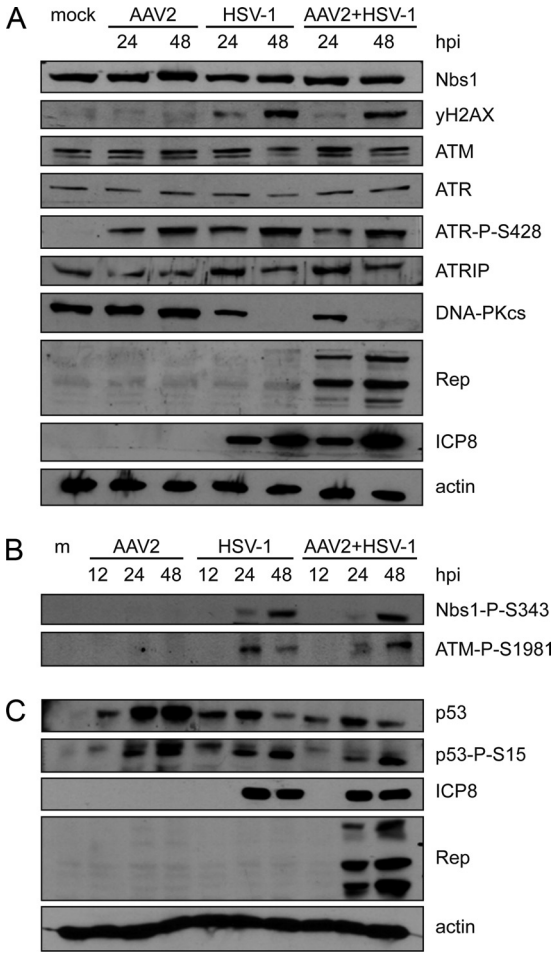



**FIG 1** DNA damage signaling induced by viral infection. (A to C) WB analysis of infected cell lysates. MO59J Fus1 cells were mock infected (m), infected with AAV2 alone (MOI, 2,000) or HSV-1 alone (MOI, 1.5), or coinfected with AAV2 (MOI, 2,000) and HSV-1 (MOI, 1.5) and harvested at the indicated times postinfection. Total proteins were extracted, separated by SDS-PAGE, blotted onto nitrocellulose membrane, and analyzed with the antibodies indicated. Actin served as a loading control; detection of HSV-1 ICP8 and AAV2 Rep was used as an infection control.

strates that the staining requires ATM activity but does not necessarily represent ATM itself.

Total ATR levels were also comparable in virus-infected cells and mock-infected cells (Fig. 1A), but phosphorylated ATR-P-S428 was present only in cells infected with either HSV-1 or AAV2 alone or coinfected with both viruses (Fig. 1A), and it colocalized with approximately 85% of the HSV-1 RCs and approximately 87% of the AAV2 RCs (Fig. 3B). The observed phosphorylation of ATR in cells infected with HSV-1 alone was surprising, as it appeared to be in contrast to previous studies which did not find HSV-1-induced activation of ATR (58, 94). We therefore further investigated the phosphorylation/activation of ATR by (i) testing the specificity of the ATR-P-S428 antibody (70), (ii) monitoring ATRIP, a protein that is important for the recruitment of ATR to sites of DNA damage (89, 106), and (iii) analyzing the phosphorylation status of the ATR target Chk1. The results of these experiments can be summarized as follows. (i) Immunoprecipitation with an ATR-specific antibody resulted in ATR-P-S428-specific

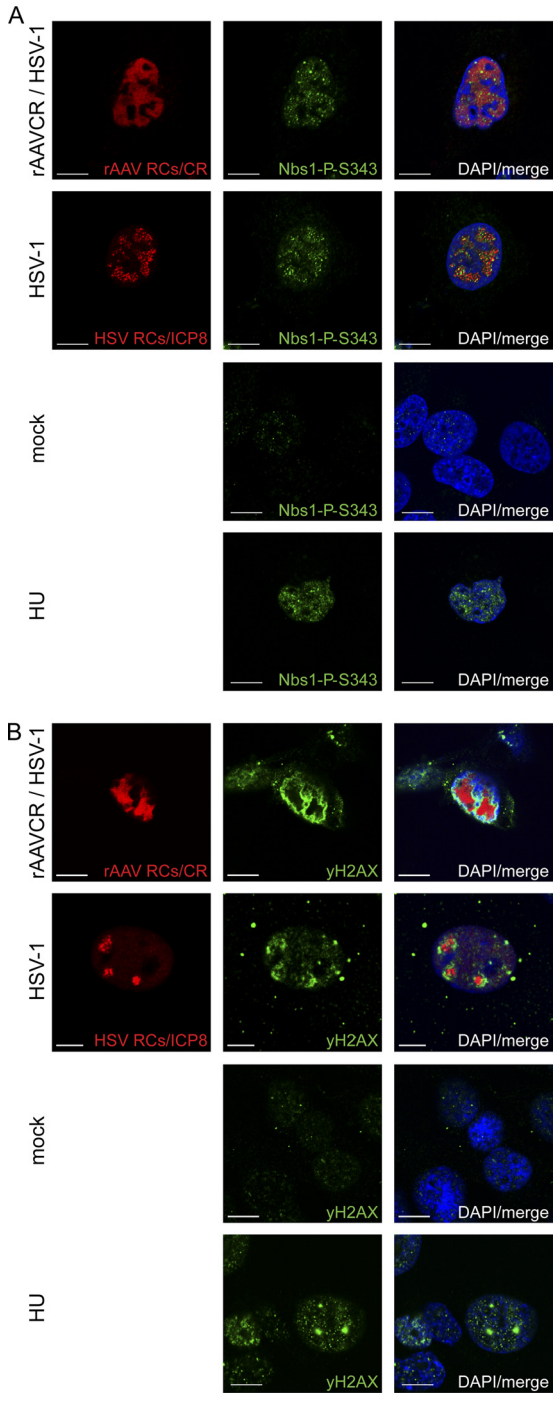



**FIG 2** Activation of primary DDR proteins. MO59J Fus1 cells were mock infected, infected with HSV-1 (MOI, 1.5), or coinfected with rAAVCR (MOI, 250) and HSV-1 (MOI, 1.5). After 24 h, cells were fixed and processed for IF analysis. rAAVCR RCs (AAV RCs) were visualized by binding of the rAAVCR-encoded mCherry-Rep68/78 fusion protein (CR) to AAV DNA (red). HSV-1 RCs were visualized with a primary antibody specific for the HSV-1 major DNA binding protein ICP8 and an AF594-labeled secondary antibody (red). Cells treated with HU (3 mM) served as a DDR control. To identify phosphorylated Nbs1 (A) and H2AX (B), cells were stained with antibodies specific for Nbs1-P-S343 or H2AX-P-S139 (γH2AX) and an FITC-labeled secondary antibody (green). DAPI was used to stain cellular DNA. Images were taken using a CLSM and represent a single optical *z* slice of the nuclei. Scale bars, 10 $\mu$m.

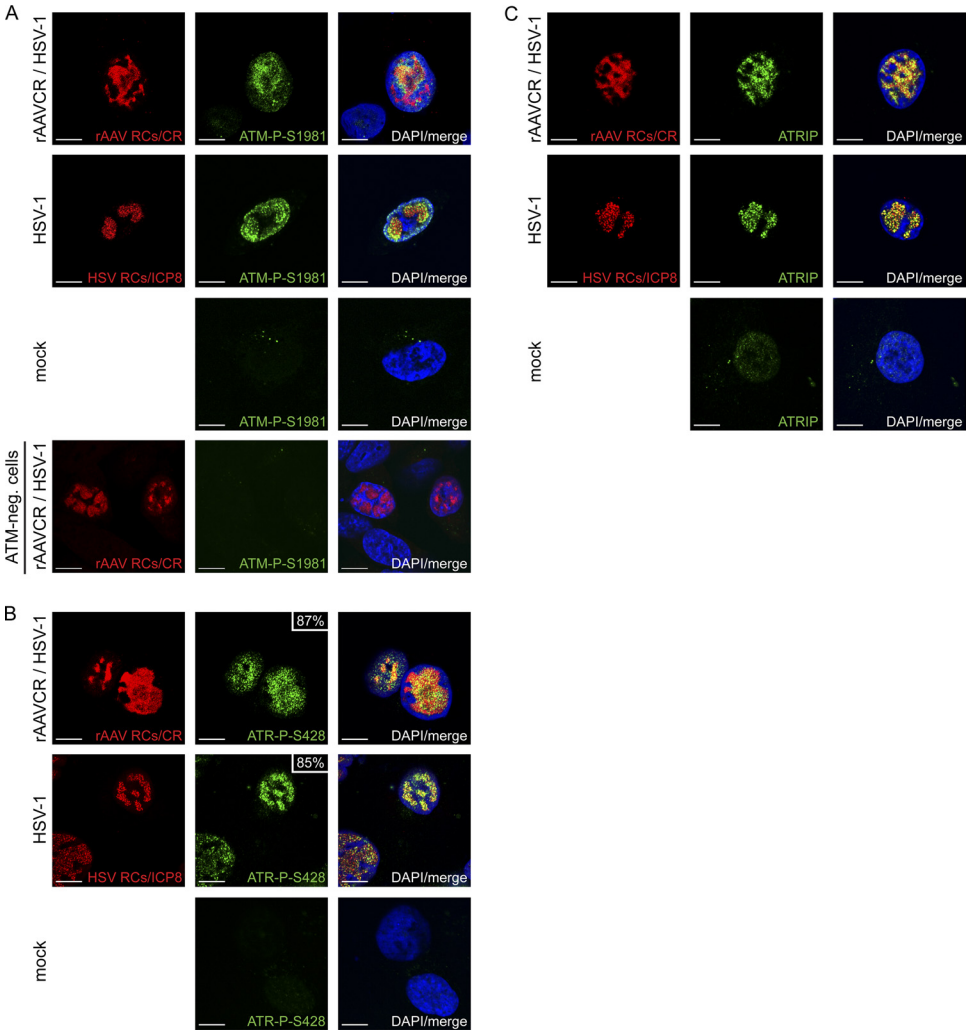



**FIG 3** Recruitment of ATM-P-S1981, ATR-P-S428, and ATRIP into HSV-1 and AAV2 RCs. MO59J Fus1 cells were infected and processed for IF, and viral RCs (red) were visualized as described in the legend to Fig. 2. Cells were stained with an antibody specific for ATM-P-S1981 (A), ATR-P-S428 (B), or ATRIP (C) and an FITC-labeled secondary antibody (green). DAPI was used to stain cell nuclei. In panel A, cells deficient for ATM (AT-22 IJE T) served as a control for the phosphospecific ATM antibody. In panel B, the percentage of ATR-P-S428 in viral RCs is indicated. Scale bars, 10 $\mu$m.

bands in cells infected with HSV-1 and/or AAV2 but not in mock-infected cells (see Fig. S1A in the supplemental material). (ii) ATRIP was detected in both mock-infected cells and virus-infected cells (Fig. 1A), where it colocalized with AAV2 and HSV-1 RCs (Fig. 3C). (iii) WB analysis revealed no phosphorylation of the ATR target Chk1 at S345 (see Fig. S1B in the supplemental material) and S317 (data not shown) in infected cells. From the results described above, it seems that although ATR is phosphorylated in infected cells, the kinase is not activated and therefore not able to transmit DDR signaling via its downstream target, Chk1. Supporting this, Nam and Cortez (62) recently showed that phosphorylation of ATR on S428 is not indicative of ATR activity and cannot be inhibited by the commonly used ATR inhibitor caffeine. Moreover, Liu et al. reported that the mutation of S428 to A428 did not prevent the phosphorylation of Chk1 in response to replicative stress (54).

**HSV-1 ICP0-dependent degradation of DNA-PKcs is delayed during coinfection with AAV2.** HSV-1 ICP0 is known to induce proteasomal degradation of DNA-PKcs (48, 67), which, together with Ku70 and Ku80, forms DNA-PK, the third main PIKK besides ATM and ATR (reviewed in reference 55). Consistent with these reports, our WB results displayed loss of DNA-PKcs by 48 h after infection with HSV-1 or coinfection with HSV-1 and AAV2 (Fig. 1A). Accordingly, at the single-cell level, DNA-PKcs was not detected simultaneously with either the HSV-1 immediate-early (IE) protein ICP0 or HSV-1 early (E) protein ICP8 in cells infected with HSV-1 alone. However, DNA-PKcs was readily detected in cells infected with rHSV-1ΔICP0 (Fig. 4A). Interestingly, IF analysis revealed that DNA-PKcs colocalized with HSV-1 proteins ICP0 and ICP8 in 80% and 76% of the AAV RCs, respectively (Fig. 4B). This indicates that HSV-1 ICP0-mediated DNA-PKcs degradation in coinfected cells is not as efficient as in cells that are infected with HSV-1 alone, even if the intensity of DNA-PKcs staining in large AAV RCs is lower than that in small AAV RCs (Fig. 4B). This possibility was further investigated by flow cytometry data. Cells infected with HSV-1 or coinfected with HSV-1 and AAV2 were identified using rHSV-1 and rAAV expressing ICP4-EYFP or EGFP, respectively. Similar

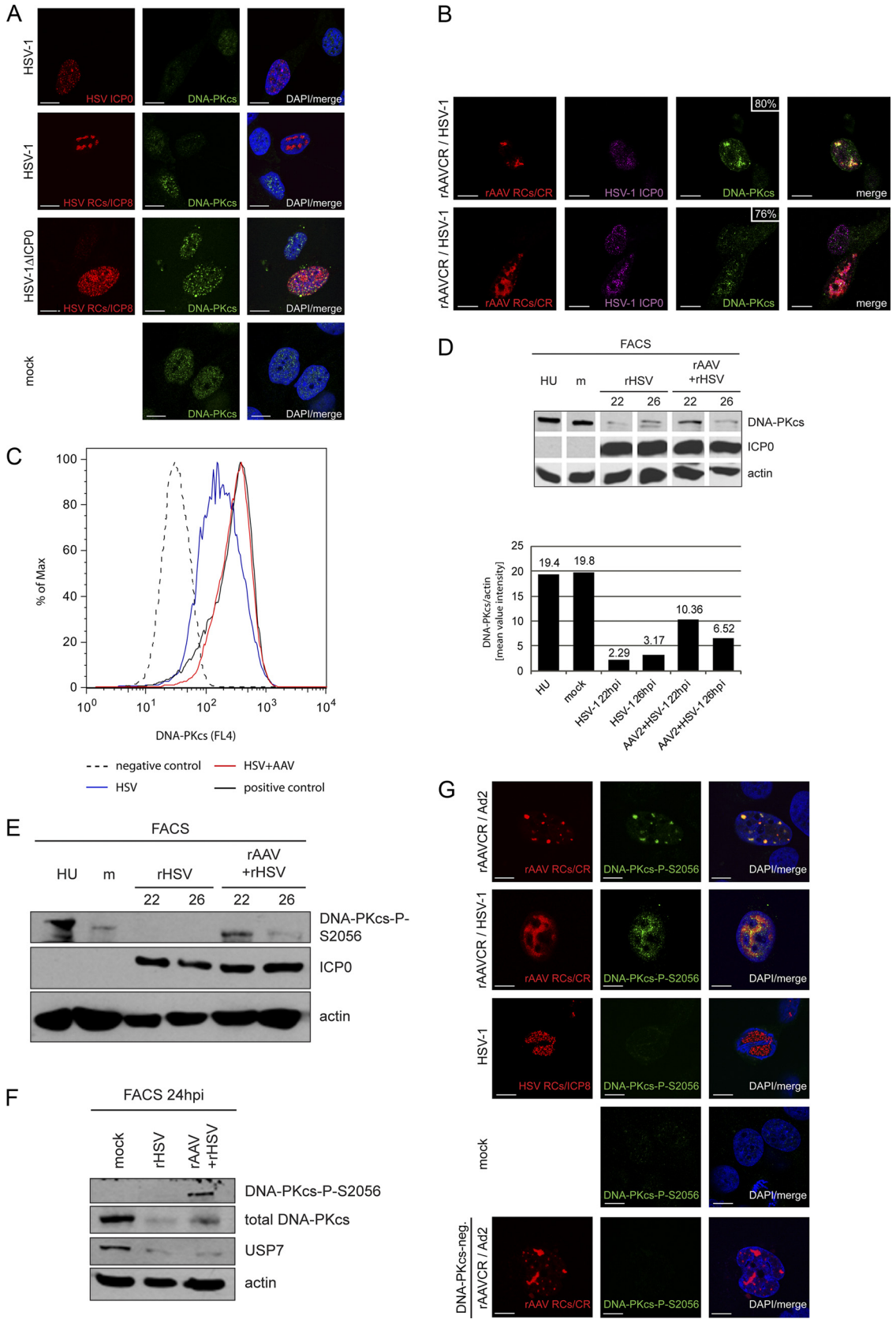



**FIG 4** Activation and delayed degradation of DNA-PKcs in cells coinfected with HSV-1 and AAV2. (A) MO59J Fus1 cells were infected with HSV-1 (MOI, 1.5) or HSV-1ΔICP0 (MOI, 0.9) or mock infected. After 24 h, cells were fixed and processed for IF analysis. HSV-1 infection was detected using an antibody specific for ICP0 or ICP8 and an AF594-labeled secondary antibody (red). Additionally, cells were stained with an antibody specific for DNA-PKcs and an FITC-labeled

to the results obtained with wild-type HSV-1 (strain F), DNA-PKcs was also degraded in cells infected with autofluorescent recombinant HSV-1 (strain17; Fig. 4C). In contrast, the fluorescence intensity of DNA-PKcs staining in cells coinfected with wild-type HSV-1 and AAV2 was the same as that in mock-infected cells (Fig. 4C). At the chosen MOIs, the ratio of cells that show AAV RCs at 24 hpi is only approximately 10%, while 24 h after infection with HSV-1 alone, more than 40% of the cells contain HSV-1 RCs. This may explain why in the WB analysis of total cell lysates shown in Fig. 1, the degradation of DNA-PKcs appeared equally efficient in HSV-1-infected cells and in coinfected cells while, on the single-cell level (Fig. 4B and C), DNA-PKcs degradation appeared to be less efficient in coinfected cells.

To further examine this observation, we performed WB analysis of sorted cells at 22 and 26 hpi (Fig. 4D and E). Cells were infected with rHSV-1 ICP4-ECFP or coinfected with rAAVCR and rHSV-1 ICP4-ECFP. HSV-1-infected cells were sorted based on the expression of ICP4-ECFP, and cells that contained rAAV RCs were sorted based on the expression of mCherry-Rep. Sorted mock-infected cells served as a control. Mock-infected and HU-treated cells served as an activation control for DNA-PKcs. Quantification of the WB assay shown in Fig. 4D revealed 3-times-higher levels of DNA-PKcs at 22 hpi during AAV replication than in cells infected with HSV-1 alone (Fig. 4D). At 26 hpi, the intensity of the DNA-PKcs staining was also decreased in coinfected cells compared to that seen at 22 hpi but still approximately 2 times as high as that in cells infected with HSV-1 alone at 26 hpi. Moreover, phosphorylation of DNA-PKcs at S2056 was detected only in cells coinfected with both viruses (Fig. 4E and F); however, pDNA-PKcs levels also decreased in coinfected cells over time (Fig. 4E). Staining for the cellular deubiquitinase ubiquitin-specific peptidase 7 (USP7) at 24 hpi, which is another known target of HSV-1 ICP0-induced proteasomal degradation (12), revealed that the AAV-mediated delay of the ICP0 function was specific for DNA-PKcs, as the degradation of USP7 was equally efficient in cells infected with HSV-1 alone and in coinfected cells (Fig. 4F).

Next, we analyzed the localization of DNA-PKcs-P-S2056 within infected cells. As positive and negative controls, we coinfected DNA-PKcs-positive cells and DNA-PKcs-deficient cells with Ad and AAV2, which are known to activate DNA-PKcs and recruit it into RCs (75). While DNA-PKcs-P-S2056 staining was readily detected in cells coinfected with AAV2 and either Ad2 or HSV-1, where it also colocalized with AAV2 RCs, we did not observe staining in cells that contained HSV-1 RCs (Fig. 4G).

**Phosphorylation of Chk2 and p53.** To explore a potential DNA-PK-mediated signaling to downstream targets in coinfected cells, we assessed virus-induced phosphorylation of Chk2 and p53 in normal cells and in cells defective for either DNA-PKcs (MO59J Fus9) or ATM (AT22 IJE-T pEBS7). HSV-1 has previously been shown to induce the phosphorylation of Chk2 in an ATM-dependent manner (51, 76). Our results are in line with these reports, as in normal cells (data not shown) and in DNA-PKcs-deficient cells (Fig. 5A), Chk2 was phosphorylated at 24 h after infection with HSV-1, and this also occurred in cells coinfected with HSV-1 and AAV2. However, in ATM-deficient cells, Chk2 was phosphorylated only in cells coinfected with both viruses but not in cells infected with HSV-1 alone (Fig. 5B). This suggests that in these cells, Chk2 phosphorylation was possibly facilitated by the AAV2-mediated delayed degradation of DNA-PKcs. Also consistent with previous observations, we detected that HSV-1 (13) and AAV2 (68) induced the stabilization of p53 (Fig. 1C). Additionally, we observed that p53 was phosphorylated at S15 in cells infected with either HSV-1 or AAV2 or coinfected with both viruses (Fig. 1C) and that p53-P-S15 was recruited into both HSV-1 RCs and AAV2 RCs (data not shown). p53-P-S15 was also detected in DNA-PKcs-deficient cells infected with HSV-1 alone or coinfected with HSV-1 and AAV2 (Fig. 5A); in these cells, phosphorylation is likely ATM mediated. However, as opposed to the situation in normal cells infected with AAV2 alone, p53-P-S15 was not detected in DNA-PKcs-deficient cells infected with AAV2 alone (Fig. 5A), indicating that in the absence of the helper virus, AAV2-induced phosphorylation of p53 is DNA-PK dependent. In ATM-deficient cells, p53 was phosphorylated only when cells were infected with rHSV-1ΔICP0 or coinfected with AAV2 and HSV-1 (Fig. 5C) and not in cells infected with HSV-1 alone (Fig. 5C), indicating again that in coinfected cells, p53 phosphorylation was supported by the delayed degradation of DNA-PKcs.

**Phosphorylation of RPA.** The trimeric RPA complex composed of RPA70, RPA32, and RPA14 is involved in DNA repair, as well as replication and transcriptional regulation of both cellular and viral DNAs (10, 97). RPA has a strong affinity for single-stranded DNA (98) and was shown to be a component of Ad-supported AAV2 RCs (79). Upon DNA damage, RPA32 becomes phosphorylated at several residues (reviewed in reference 107). It was suggested that phosphorylation at S33 is mediated by ATR,

secondary antibody (green). DAPI was used to stain cellular DNA. Scale bars, 10 μm. (B) IF analysis of MO59J Fus1 cells at 24 h after coinfection with rAAVCR (MOI, 250) and HSV-1 (MOI, 1.5). rAAVCR RCs (red) were visualized as described in the legend to Fig. 2. Additionally, cells were stained with antibodies specific for ICP0 or ICP8 and an AF405-labeled secondary antibody (purple) and with an antibody specific for DNA-PKcs and an FITC-labeled secondary antibody (green). The percentage of DNA-PKcs in rAAVCR RCs is indicated. Scale bars, 10 μm. (C) Flow cytometric analysis of infected cells. DNA-PKcs-positive HCT116 cells were mock infected (positive control), infected with rHSV-1ICP4EYFP (MOI, 1.5), or coinfected with HSV-1 (MOI, 1.5), AAV2 (MOI, 250), and rAAVGFP (MOI, 250). HCT116 cells negative for DNA-PKcs served as a negative control. Cells were fixed at 20 h postinfection and stained with a DNA-PKcs-specific monoclonal antibody and a Cy5-labeled secondary antibody. DNA-PKcs was analyzed in HSV-1-infected cells positive for EYFP-ICP4 (HSV) and in coinfected cells positive for EGFP (HSV+AAV). A minimum of 80,000 events were scored for each sample. Graphs were overlaid to show the fluorescence shift of HSV-1-infected populations. (D and E) WB analysis of AT22 IJE-T cells sorted for productive HSV-1 and AAV2 infection at 22 and 26 hpi. Cells were mock infected, infected with rHSV-1vECFP-ICP4 (rHSV; MOI, 2), or coinfected (rHSV + rAAV) with rHSV-1vECFP-ICP4 (MOI, 2) and rAAV2CR (MOI, 4,000). Lysates of sorted cells were processed for WB analysis and stained with the antibodies indicated. Quantification of WB band intensities was done with a Gel Doc system using Quantity One software (version 4.6.1; Bio-Rad, Hercules, CA). (F) WB analysis of AT22 IJE-T cells sorted for productive HSV-1 and AAV2 infection at 24 hpi. Cells were mock infected, infected with rHSV-1vECFP-ICP4 (rHSV; MOI, 4), or coinfected (rHSV + rAAV) with rHSV-1vECFP-ICP4 (MOI, 4) and rAAV2CR (MOI, 4,000). Lysates of sorted cells were processed for WB analysis and stained with the antibodies indicated. (G) MO59J Fus1 cells were mock infected, infected with HSV-1 (MOI, 1.5), or coinfected with rAAVCR (MOI, 250) and either HSV-1 (MOI, 1.5) or Ad2 (MOI, 12.5). After 24 h, cells were fixed and processed for IF analysis. rAAVCR and HSV-1 RCs (red) were visualized as described in the legend to Fig. 2. DNA-PKcs phosphorylation was detected with an antibody specific for DNA-PKcs-P-S2056 and an FITC-labeled secondary antibody (green). As a negative control, MO59J Fus9 DNA-PKcs-negative cells were coinfected with rAAVCR (MOI, 250) and Ad2 (MOI, 12.5) and stained for pDNA-PKcs. DAPI was used to stain cellular DNA. Scale bars, 10 μm.

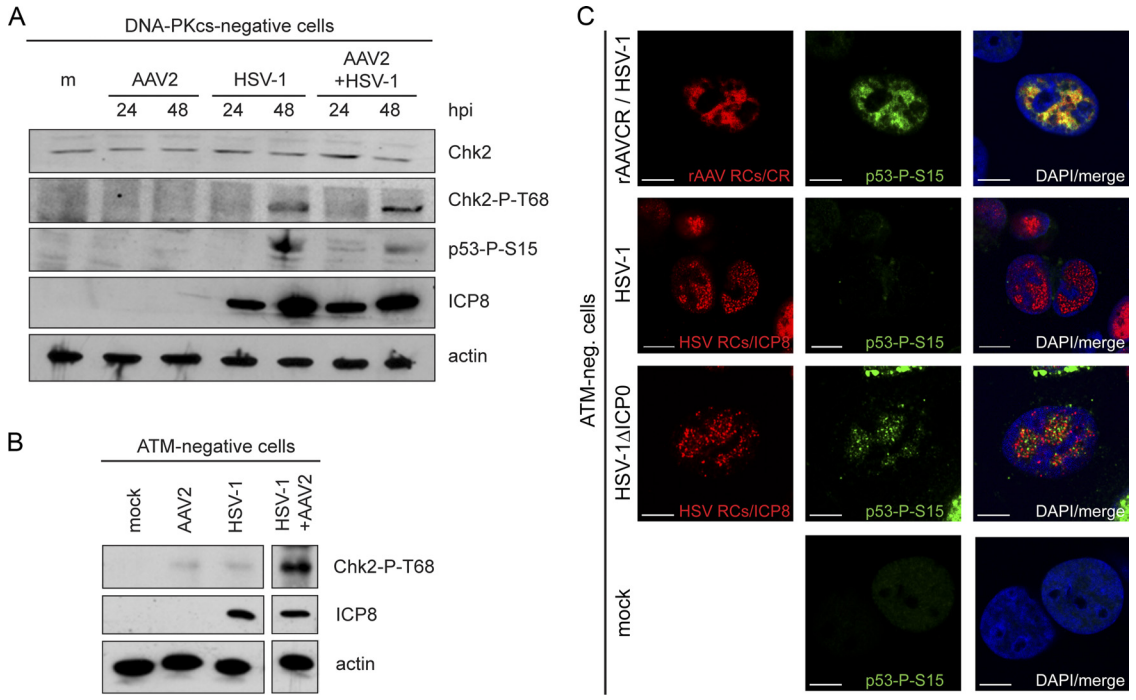



**FIG 5** DNA-PKcs- and ATM-dependent activation of Chk2 and p53 upon viral infection. (A) WB analysis of DNA-PKcs-deficient MO59J Fus9 cells. Cells were mock infected (m), infected with AAV2 (MOI, 2,000) or HSV-1 (MOI, 1.5), or coinfected with AAV2 (MOI, 2,000) and HSV-1 (MOI, 1.5). Total proteins were extracted at the indicated times postinfection and subjected to WB analysis using antibodies specific for actin (loading control), ICP8 (HSV-1 infection control), Chk2, Chk2-P-T68, and p53-P-S15. (B) Immunoprecipitation and WB analysis of ATM-negative AT22 IJE-T cells at 24 h after mock infection or infection with AAV2 (MOI, 2,000), HSV-1 (MOI, 1.5), AAV2 (MOI, 2,000), and HSV-1 (MOI, 1.5). Lysates were analyzed using the antibodies indicated. (C) IF analysis of ATM-negative AT22 IJE-T cells at 24 h after infection with HSV-1 (MOI, 1.5) and rAAVCR (MOI, 250), HSV-1 (MOI, 1.5), or HSV-1ΔICP0 (MOI, 0.9) or mock infection. rAAVCR and HSV-1 RCs (red) were visualized as described in the legend to Fig. 2. p53 activation was detected with an antibody specific for p53-P-S15 and an FITC-labeled secondary antibody (green). DAPI was used to stain cellular nuclei. Scale bars, 10 $\mu$m.

while phosphorylation at S4/8 is mediated in a DNA-PK-dependent manner (4, 104). Here we examined the spatial localization of the RPA subunit RPA32 and its phosphorylation at S4/8 in infected cells. Our results show that total RPA32 is recruited into HSV-1 and AAV2 RCs (data not shown) and that its expression levels were not altered by virus infection (Fig. 6A). WB analysis of cells productively infected with either HSV-1 or AAV2 revealed phosphorylation of RPA32 at Ser4/8 only upon AAV2 replication (Fig. 6A). More precisely, RPA32-P-S4/8 clearly colocalized with approximately 70% of the small and large AAV2 RCs but with only 21% of the small and 11% of the large HSV-1 RCs (Fig. 6B and C). UV-treated cells served as a positive control (Fig. 6B). Moreover, accumulation of RPA32-P-S4/8 was observed only in AAV2 RCs of DNA-PKcs-positive cells (Fig. 6D). In summary, these data show that coinfection with AAV2 and HSV-1 induces DNA-PKcs-dependent phosphorylation of RPA32 at S4/8.

## DISCUSSION

In this study, we compared the phosphorylation status and spatial organization of DDR proteins between cells infected with HSV-1 alone and cells coinfected with HSV-1 and AAV2. WB and IF analyses demonstrated that HSV-1 and AAV2 coinfection induced a strong DDR (Table 1 and Fig. 7). Specifically, HSV-1-supported AAV2 replication induced the phosphorylation of Nbs1 and H2AX, as well as ATM and its substrates Chk2 and p53. These responses are similar to those induced by HSV-1 infection alone, as observed here and in previous studies (13, 49, 67, 76, 81, 95). We also detected phosphorylation of ATR and recruitment of pATR along with its binding partner ATRIP into HSV-1-supported AAV2 RCs, as well as into HSV-1 compartments, although kinase activity of ATR was blocked upon HSV-1 and AAV2 replication, as we did not detect phosphorylation of Chk1 at ATR target sites S317 and S345. Our results are similar to those of a previous study showing that ATR, although recruited together with ATRIP into HSV-1 RCs, is unable to activate Chk1. In addition, Mohni et al. showed that both ATR and ATRIP, even if not activated, contribute to efficient HSV-1 infection (58).

Due to the strong affinity of RPA32 for single-stranded DNA (102, 107) and its function in recruiting the ATRIP-ATR complex to sites of DNA damage (7, 26, 106), it is not surprising that we and others (65, 79) detected RPA32 in HSV-1-supported AAV2 RCs. In HSV-1-infected cells, RPA32 colocalizes with the HSV-1 major single-stranded DNA binding protein ICP8 (81) in HSV-1 RCs (82, 93). Replicating HSV-1 DNA contains stretches of single-stranded DNA (39) which may recruit RPA32 into HSV-1 RCs also independently of ICP8. Single-stranded DNA binding proteins such as RPA32 are proposed to stimulate AAV2 DNA replication (64, 79), e.g., by protecting the single-stranded replication products from nucleases (63) and by enhancing the binding and nicking of Rep proteins at the replication origins (79).

HSV-1 has been demonstrated to induce the degradation of DNA-PKcs in an ICP0-dependent manner (48, 67). Although WB analysis of total infected cells displayed a similar degradation of DNA-PKcs, in cells infected with HSV-1 or coinfected with HSV-1

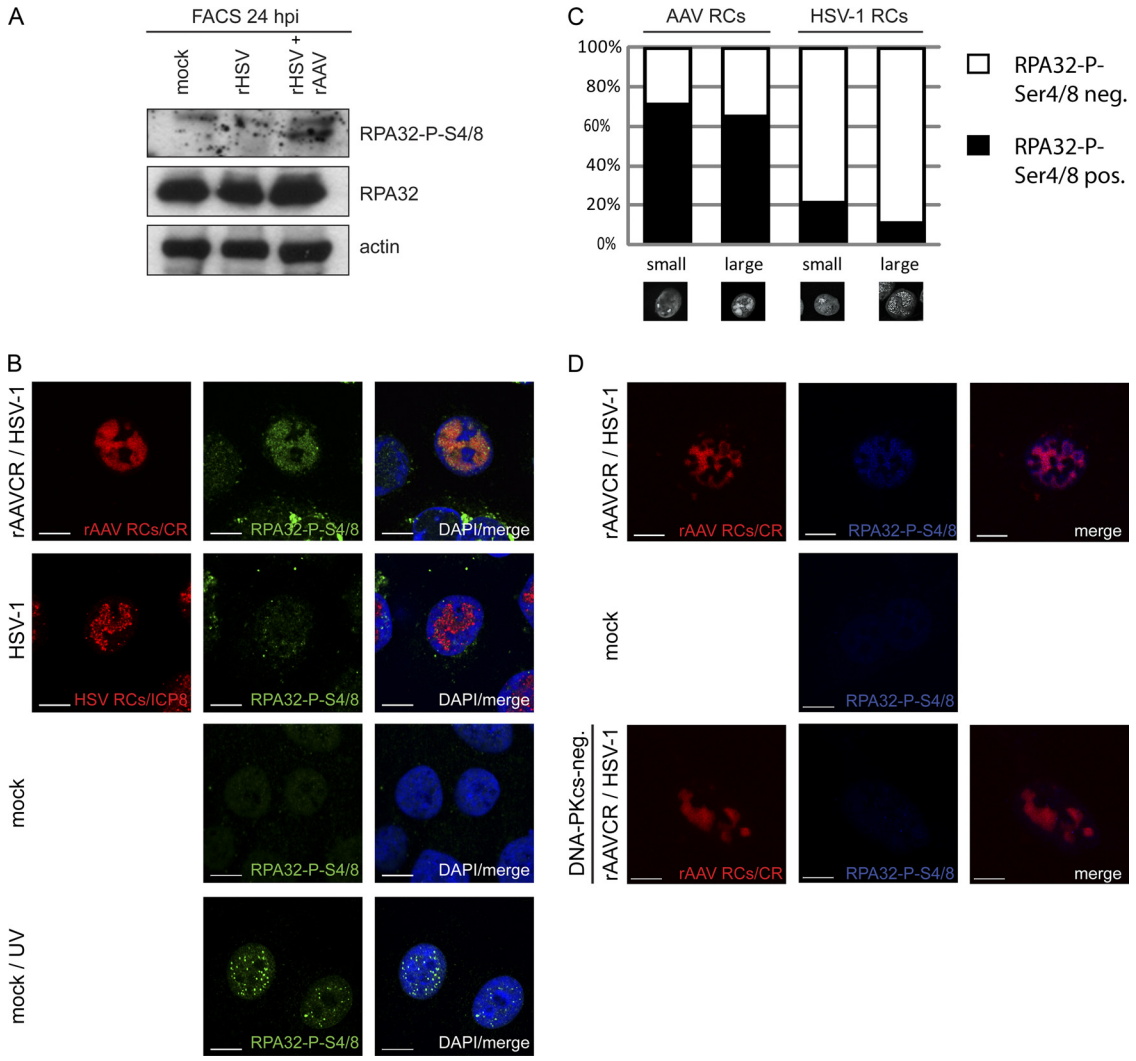



**FIG 6** Phosphorylation of RPA32 upon AAV2 and HSV-1 replication. (A) WB analysis of sorted AT22 IJE-T cells at 24 hpi. Cells were mock infected, infected with rHSV-1vECFP-ICP4 (rHSV; MOI, 4), or coinfected (rHSV + rAAV) with rHSV-1vECFP-ICP4 (MOI, 4) and rAAV2CR (MOI, 4,000). Sorted cells were subjected to WB analysis and analyzed with the antibodies indicated. (B) IF analysis of U2OS cells after infection with HSV-1 (MOI, 1.5) or rAAVCR (MOI, 250) and HSV-1 (MOI, 1.5) or mock infection at 24 h. UV-treated cells (10 J/m$^2$) served as a positive control. rAAVCR and HSV-1 RCs (red) were visualized as described in the legend to Fig. 2. To detect phosphorylated RPA32, cells were stained with an antibody specific for RPA32-P-S4/8 and an FITC-labeled secondary antibody (green). Cellular DNA was stained with DAPI. Scale bars, 10 $\mu$m. (C) Quantification of RPA32-P-S4/8 colocalization with small and large AAV2 or HSV-1 RCs in U2OS cells. Fifty cells per sample were counted. Black columns, RPA32-P-S4/8-positive viral RCs; open columns, RPA32-P-S4/8 negative viral RCs. (D) IF analysis of MO59J Fus1 or Fus9 (DNA-PKcs-negative) cells at 24 h after mock infection or coinfection with HSV-1 (MOI, 1.5) and rAAVCR (MOI, 250). rAAVCR RCs (red) were visualized as described in the legend to Fig. 2. Cells were stained with an antibody specific for RPA32-P-S4/8 and an AF405-labeled secondary antibody (blue). Scale bars, 10 $\mu$m.

and AAV2, a striking difference was observed at the single-cell level, as well as in cells productively infected with HSV-1 and AAV2 captured by FACS. Flow cytometry, IF analysis, and WB analysis of sorted cells revealed an AAV2-mediated delay in the HSV-1-dependent degradation of DNA-PKcs, as well as AAV2-induced phosphorylation of DNA-PKcs. The observed discrepancy in the results of WB assays of total cell lysates and analyses on the single-cell level is likely due to the fact that, under the chosen experimental conditions, only approximately 10% of the cells supported AAV2 replication. In order to estimate the extent of the delay in DNA-PKcs degradation, we covisualized DNA-PKcs and the HSV-1 IE and E proteins ICP0 and ICP8, respectively, which allowed monitoring of the progression of HSV-1 infection. While DNA-PKcs and ICP0 or ICP8 were not codetected in cells infected with HSV-1 alone, DNA-PKcs and ICP0 or ICP8 were consistently codetected in cells coinfected with HSV-1 and AAV2, and they colocalized with AAV2 RCs. As we did not observe an AAV2-mediated inhibition of ICP0 in this study (see Fig. S1B in the supplemental material) or in a previous study (31), we speculate that AAV2 replication can prevent the ICP0-dependent degradation of DNA-PKcs (48, 67), e.g., by shielding DNA-PKcs in viral RCs. We can exclude the possibility that AAV2 replication directly inhibits the E3 ubiquitin ligase activity of ICP0 because USP7, another target of ICP0-mediated proteasomal degradation (12), was rapidly degraded in coinfected cells. In addition, we can exclude a possible role for USP7 in preventing the degradation of

**TABLE 1** DNA damage signaling in cells infected with HSV-1 or coinfected with HSV-1 and rAAV2[a]

| Protein | HSV-1–rAAV2 coinfection | | | | HSV-1 infection | | | |
|---|---|---|---|---|---|---|---|---|
| | Inhibition | Induction | Phosphorylation | Localization | Inhibition | Induction | Phosphorylation | Localization |
| ATM | | | + | NUC[c] + RCs | | | + | RCs |
| ATR | − | − | + | RCs | − | − | + | RCs |
| ATRIP | − | + | ND[d] | RCs | − | + | ND | RCs |
| **DNA-PKcs** | **+**[b] | **−** | **+** | **RCs** | **+** | **−** | **−** | **−** |
| USP7 | + | − | ND | RCs | + | − | ND | ND |
| H2AX | ND | ND | + | NUC | ND | ND | + | NUC |
| NBS1 | − | − | + | RCs | − | − | + | RCs |
| **RPA32 (P-Ser4/8)** | **−** | **−** | **+** | **RCs** | **−** | **−** | **−** | **RCs** |
| p53 | − | + | + | RCs | − | + | + | RCs |
| Chk1 | − | − | − | RCs | − | − | − | RCs |
| Chk2 | − | − | + | RCs | − | − | + | RCs |

[a] Shown is a summary of data from WB analysis, IF analysis, and flow cytometry. Bold, AAV-induced modulation of DDR.
[b] Inhibition delayed.
[c] NUC, nucleus.
[d] ND, not done.

DNA-PKcs by deubiquitinating (45) DNA-PKcs. Experiments performed with ATM-deficient cells revealed that the AAV2-mediated delay of DNA-PKcs degradation affected downstream signaling via Chk2 and p53. In these cells, coinfection with both viruses resulted in a DDR comparable to that induced by infection with HSV-1ΔICP0, including phosphorylation of p53. Consistent with previous data (51, 76), infection of ATM-deficient cells with HSV-1 alone resulted in a broad reduction of DDR signaling with undetectable levels of Chk2-P-T86 and p53-P-S15. We therefore hypothesize that in coinfected ATM-deficient cells, DNA-PK is responsible for the phosphorylation of Chk2 and p53 (Fig. 7). A role for DNA-PK in the phosphorylation of Chk2 and p53 has previously been reported (49, 50, 85, 86, 88). In fact, here we demonstrate that DNA-PK is essential for the activation of p53 in cells infected with AAV2 alone (Fig. 1C and 5A).

Recently, we reported hyperphosphorylation of RPA32 at the DNA-PK target site S4/8 in cells transfected with a plasmid encoding the AAV2 Rep68 or Rep78 protein (30). In the present study, we detected phosphorylation of RPA32 at S4/8 and recruitment of RPA32-P-S4/8 into HSV-1-supported AAV2 RCs. In contrast, HSV-1 infection alone did not induce phosphorylation of RPA32 at S4/8. Similar to the phosphorylation of Chk2 and p53 in coinfected cells, phosphorylation of RPA32 at S4/8 may also be a consequence of the delayed degradation of DNA-PKcs. Further support for this theory comes from the fact that DNA-PK has previously been demonstrated to phosphorylate RPA32 at S4/8 (4, 11, 104).

In a comparison of our results obtained with coinfected cells with those of previous studies examining the DDR signaling in cells infected with HSV-1 alone (13, 51, 76, 96) or Ad2 alone (17, 78) or coinfected with AAV2 and Ad2 (25, 75), it is remarkable that although Ad and HSV-1 by themselves induce very different DDRs, in the presence of AAV2, the induction and activation patterns of DDR proteins are more similar. It seems that during AAV2 replication, the cellular DDR is modulated toward DNA-PK-dependent signaling, which might have positive and/or negative impacts on AAV replication and transduction, depending on the cellular environment and the viral genome structure (23, 25, 75). Further experiments will address the question of whether this

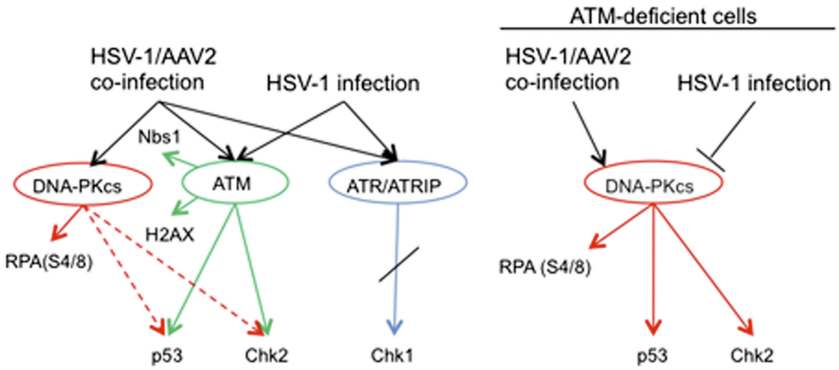



**FIG 7** Models of the DDR signaling induced by HSV-1 replication and HSV-1-supported AAV replication. The analysis of the DDRs in ATM-deficient, DNA-PKcs-deficient, and normal cells supports the following models. Infection of cells with HSV-1 induces phosphorylation of ATM and ATR and signaling to their targets Nbs1, H2AX, Chk2, and p53 but not to the ATR target Chk1. In cells infected with HSV-1 alone, DNA-PKcs is rapidly degraded in an HSV-1 ICP0-dependent manner and no DNA-PKcs-mediated signaling occurs. In contrast, coinfection with HSV-1 and AAV2 induces the activation of DNA-PKcs, which enables the phosphorylation of RPA32 at S4/8. Further support for the activation of DNA-PKcs in coinfected cells comes from experiments performed with ATM-deficient cells, as in the absence of ATM, HSV-1 and AAV2 coinfection still induced the phosphorylation of p53, Chk2, and RPA32 at S4/8. In ATM-deficient cells infected with HSV-1 alone, the DDR signaling is broadly reduced, with undetectable levels of Chk2-P-T86 and p53-P-S15 (51, 76).

modulation can enhance HSV-1-supported AAV2 replication and/or inhibit helper virus replication.

## ACKNOWLEDGMENTS

We thank P. Beard (ETH, Lausanne, Switzerland) for helpful discussions and A. Nicolas (INSERM, Lyon, France) for critically reading the manuscript. We are also grateful to E. Hendrickson, T. Melendy, Y. Shiloh, and P. Nghiem for providing cell lines and B. Roizman, N. Stow, R. D. Everett, H. Buening, U. Greber, and M. Linden for providing viruses.

This work was supported by Swiss National Science Foundation grant 31003A_124938 to C.F. D.L.G. is supported by fellowships from the Swiss National Science Foundation and the Swiss Foundation for Grants in Biology and Medicine (PBZHP3-122925 and PASMP3-132554). A.S. and N.J. are supported by INSERM and the Association Française contre les Myopathies (AFM). Work in the Weitzman laboratory was partially supported by a Pioneer Developmental Chair from the Salk Institute and by NIH grants CA97093 and AI43341 (M.D.W.).

## REFERENCES

1. **Adeyemi RO, Landry S, Davis ME, Weitzman MD, Pintel DJ.** 2010. Parvovirus minute virus of mice induces a DNA damage response that facilitates viral replication. PLoS Pathog. **6**:e1001141.
2. **Ahn JY, Schwarz JK, Piwnica-Worms H, Canman CE.** 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res. **60**:5934–5936.
3. **Alazard-Dany N, et al.** 2009. Definition of herpes simplex virus type 1 helper activities for adeno-associated virus early replication events. PLoS Pathog. **5**:e1000340.
4. **Anantha RW, Vassin VM, Borowiec JA.** 2007. Sequential and synergistic modification of human RPA stimulates chromosomal DNA repair. J. Biol. Chem. **282**:35910–35923.
5. **Antoni BA, et al.** 1991. Adeno-associated virus Rep protein inhibits human immunodeficiency virus type 1 production in human cells. J. Virol. **65**:396–404.
6. **Bakkenist CJ, Kastan MB.** 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature **421**:499–506.
7. **Ball HL, Myers JS, Cortez D.** 2005. ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation. Mol. Biol. Cell **16**:2372–2381.
8. **Banin S, et al.** 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science **281**:1674–1677.
9. **Berthet C, Raj K, Saudan P, Beard P.** 2005. How adeno-associated virus Rep78 protein arrests cells completely in S phase. Proc. Natl. Acad. Sci. U. S. A. **102**:13634–13639.
10. **Binz SK, Sheehan AM, Wold MS.** 2004. Replication protein A phosphorylation and the cellular response to DNA damage. DNA Repair **3**:1015–1024.
11. **Block WD, Yu Y, Lees-Miller SP.** 2004. Phosphatidyl inositol 3-kinase-like serine/threonine protein kinases (PIKKs) are required for DNA damage-induced phosphorylation of the 32 kDa subunit of replication protein A at threonine 21. Nucleic Acids Res. **32**:997–1005.
12. **Boutell C, Canning M, Orr A, Everett RD.** 2005. Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7. J. Virol. **79**: 12342–12354.
13. **Boutell C, Everett RD.** 2004. Herpes simplex virus type 1 infection induces the stabilization of p53 in a USP7- and ATM-independent manner. J. Virol. **78**:8068–8077.
14. **Buller RM, Janik JE, Sebring ED, Rose JA.** 1981. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J. Virol. **40**:241–247.
15. **Burma S, Chen BP, Chen DJ.** 2006. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA Repair **5**:1042–1048.
16. **Cahill D, Connor B, Carney JP.** 2006. Mechanisms of eukaryotic DNA double strand break repair. Front. Biosci. **11**:1958–1976.
17. **Carson CT, et al.** 2003. The Mre11 complex is required for ATM activation and the G[inf]2/M checkpoint. EMBO J. **22**:6610–6620.
18. **Cataldi MP, McCarty DM.** 2010. Differential effects of DNA double-strand break repair pathways on single-strand and self-complementary adeno-associated virus vector genomes. J. Virol. **84**:8673–8682.
19. **Chan DW, et al.** 2002. Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. Genes Dev. **16**:2333–2338.
20. **Chaturvedi P, et al.** 1999. Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene **18**: 4047–4054.
21. **Chen BP, et al.** 2005. Cell cycle dependence of DNA-dependent protein kinase phosphorylation in response to DNA double strand breaks. J. Biol. Chem. **280**:14709–14715.
22. **Chen Y, Sanchez Y.** 2004. Chk1 in the DNA damage response: conserved roles from yeasts to mammals. DNA Repair **3**:1025–1032.
23. **Choi YK, Nash K, Byrne BJ, Muzyczka N, Song S.** 2010. The effect of DNA-dependent protein kinase on adeno-associated virus replication. PLoS One **5**:e15073.
24. **Clarke PR, Allan LA.** 2009. Cell-cycle control in the face of damage—a matter of life or death. Trends Cell Biol. **19**:89–98.
25. **Collaco RF, Bevington JM, Bhrigu V, Kalman-Maltese V, Trempe JP.** 2009. Adeno-associated virus and adenovirus coinfection induces a cellular DNA damage and repair response via redundant phosphatidylinositol 3-like kinase pathways. Virology **392**:24–33.
26. **Dart DA, Adams KE, Akerman I, Lakin ND.** 2004. Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase. J. Biol. Chem. **279**:16433–16440.
27. **Daya S, Cortez N, Berns KI.** 2009. Adeno-associated virus site-specific integration is mediated by proteins of the nonhomologous end-joining pathway. J. Virol. **83**:11655–11664.
28. **Fraefel C, et al.** 2004. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. J. Virol. **78**:389–398.
29. **Giglia-Mari, G, Zotter A, Vermeulen W.** 2011. DNA damage response. Cold Spring Harb. Perspect. Biol. **3**:a000745.
30. **Glauser DL, et al.** 2010. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus Rep proteins depends on their combined DNA-binding and ATPase/helicase activities. J. Virol. **84**: 3808–3824.
31. **Glauser DL, et al.** 2007. Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication: molecular mechanisms of interaction. J. Virol. **81**:4732–4743.
32. **Guo Z, Kumagai A, Wang SX, Dunphy WG.** 2000. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. Genes Dev. **14**:2745–2756.
33. **Harper JW, Elledge SJ.** 2007. The DNA damage response: ten years after. Mol. Cell **28**:739–745.
34. **Heilbronn R, Burkle A, Stephan S, Hzur Hausen.** 1990. The adeno-associated virus *rep* gene suppresses herpes simplex virus-induced DNA amplification. J. Virol. **64**:3012–3018.
35. **Heilbronn R, et al.** 2003. ssDNA-dependent colocalization of adeno-associated virus Rep and herpes simplex virus ICP8 in nuclear replication domains. Nucleic Acids Res. **31**:6206–6213.
36. **Helleday T, Lo J, van Gent DC, Engelward BP.** 2007. DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair **6**:923–935.
37. **Hunter LA, Samulski RJ.** 1992. Colocalization of adeno-associated virus Rep and capsid proteins in the nuclei of infected cells. J. Virol. **66**: 317–324.
38. **Jackson SP, Bartek J.** 2009. The DNA-damage response in human biology and disease. Nature **461**:1071–1078.
39. **Jacob RJ, Roizman B.** 1977. Anatomy of herpes simplex virus DNA VIII. Properties of the replicating DNA. J. Virol. **23**:394–411.
40. **Jing XJ, Kalman-Maltese V, Cao X, Yang Q, Trempe JP.** 2001. Inhibition of adenovirus cytotoxicity, replication, and E2a gene expression by adeno-associated virus. Virology **291**:140–151.
41. **Jurvansuu J, Raj K, Stasiak A, Beard P.** 2005. Viral transport of DNA damage that mimics a stalled replication fork. J. Virol. **79**:569–580.
42. **Kastan MB, Lim DS.** 2000. The many substrates and functions of ATM. Nat. Rev. Mol. Cell Biol. **1**:179–186.
43. **Kastan MB, Lim DS, Kim ST, Xu B, Canman C.** 2000. Multiple signaling pathways involving ATM. Cold Spring Harb. Symp. Quant. Biol. **65**:521–526.
44. **Kleinschmidt JA, Mohler M, Weindler FW, Heilbronn R.** 1995. Se-

quence elements of the adeno-associated virus rep gene required for suppression of herpes-simplex-virus-induced DNA amplification. Virology **206**:254–262.
45. **Komander D, Clague MJ, Urbe S.** 2009. Breaking the chains: structure and function of the deubiquitinases. Nat. Rev. Mol. Cell Biol. **10**: 550–563.
46. **Lakin ND, Hann BC, Jackson SP.** 1999. The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53. Oncogene **18**:3989–3995.
47. **Lamarche BJ, Orazio NI, Weitzman MD.** 2010. The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett. **584**: 3682–3695.
48. **Lees-Miller SP, et al.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J. Virol. **70**:7471–7477.
49. **Lees-Miller SP, Sakaguchi K, Ullrich SJ, Appella E, Anderson CW.** 1992. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. Mol. Cell. Biol. **12**:5041–5049.
50. **Li J, Stern DF.** 2005. Regulation of CHK2 by DNA-dependent protein kinase. J. Biol. Chem. **280**:12041–12050.
51. **Lilley CE, Carson CT, Muotri AR, Gage FH, Weitzman MD.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc. Natl. Acad. Sci. U. S. A. **102**:5844–5849.
52. **Linden RM, Berns KI.** 2000. Molecular biology of adeno-associated viruses. Contrib. Microbiol. **4**:68–84.
53. **Liu Q, et al.** 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. **14**: 1448–1459.
54. **Liu S, et al.** 2011. ATR autophosphorylation as a molecular switch for checkpoint activation. Mol. Cell **43**:192–202.
55. **Lovejoy CA, Cortez D.** 2009. Common mechanisms of PIKK regulation. DNA Repair **8**:1004–1008.
56. **Matsuoka S, Huang M, Elledge SJ.** 1998. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science **282**:1893–1897.
57. **Matsuoka S, et al.** 2000. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc. Natl. Acad. Sci. U. S. A. **97**:10389–10394.
58. **Mohni KN, Livingston CM, Cortez D, Weller SK.** 2010. ATR and ATRIP are recruited to herpes simplex virus type 1 replication compartments even though ATR signaling is disabled. J. Virol. **84**:12152–12164.
59. **Mordes DA, Cortez D.** 2008. Activation of ATR and related PIKKs. Cell Cycle **7**:2809–2812.
60. **Morio T, Kim H.** 2008. Ku, Artemis, and ataxia-telangiectasia-mutated: signalling networks in DNA damage. Int. J. Biochem. Cell Biol. **40**: 598–603.
61. **Nada S, Trempe JP.** 2002. Characterization of adeno-associated virus rep protein inhibition of adenovirus E2a gene expression. Virology **293**: 345–355.
62. **Nam EA, Cortez D.** 2011. ATR signalling: more than meeting at the fork. Biochem. J. **436**:527–536.
63. **Nash K, Chen W, Salganik M, Muzyczka N.** 2009. Identification of cellular proteins that interact with the adeno-associated virus Rep protein. J. Virol. **83**:454–469.
64. **Ni TH, et al.** 1998. Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. J. Virol. **72**: 2777–2787.
65. **Nicolas A, et al.** 2010. Identification of Rep-associated factors in herpes simplex virus type 1-induced adeno-associated virus type 2 replication compartments. J. Virol. **84**:8871–8887.
66. **Ohnishi T, Mori E, Takahashi A.** 2009. DNA double-strand breaks: their production, recognition, and repair in eukaryotes. Mutat. Res. **669**: 8–12.
67. **Parkinson J, Lees-Miller SP, Everett RD.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J. Virol. **73**:650–657.
68. **Raj K, Ogston P, Beard P.** 2001. Virus-mediated killing of cells that lack p53 activity. Nature **412**:914–917.
69. **Reinhardt HC, Yaffe MB.** 2009. Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. Curr. Opin. Cell Biol. **21**:245–255.
70. **Robinson K, Asawachaicharn N, Galloway DA, Grandori C.** 2009. c-Myc accelerates S-phase and requires WRN to avoid replication stress. PLoS One **4**:e5951.
71. **Rommelaere J, Cornelis JJ.** 1991. Antineoplastic activity of parvoviruses. J. Virol. Methods **33**:233–251.
72. **Samulski RJ, et al.** 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J. **10**:3941–3950.
73. **Saudan P, Vlach J, Beard P.** 2000. Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. EMBO J. **19**:4351–4361.
74. **Schlehofer JR.** 1994. The tumor suppressive properties of adeno-associated viruses. Mutat. Res. **305**:303–313.
75. **Schwartz RA, Carson CT, Schuberth C, Weitzman MD.** 2009. Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. J. Virol. **83**:6269–6278.
76. **Shirata N, et al.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. J. Biol. Chem. **280**:30336–30341.
77. **Song S, et al.** 2004. DNA-dependent PK inhibits adeno-associated virus DNA integration. Proc. Natl. Acad. Sci. U. S. A. **101**:2112–2116.
78. **Stracker TH, Carson CT, Weitzman MD.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature **418**:348–352.
79. **Stracker TH, et al.** 2004. The Rep protein of adeno-associated virus type 2 interacts with single-stranded DNA-binding proteins that enhance viral replication. J. Virol. **78**:441–453.
80. **Tapia-Alveal C, Calonge TM, O'Connell MJ.** 2009. Regulation of chk1. Cell Div. **4**:8.
81. **Taylor TJ, Knipe DM.** 2004. Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J. Virol. **78**:5856–5866.
82. **Taylor TJ, McNamee EE, Day C, Knipe DM.** 2003. Herpes simplex virus replication compartments can form by coalescence of smaller compartments. Virology **309**:232–247.
83. **Tibbetts RS, et al.** 2000. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev. **14**: 2989–3002.
84. **Timpe JM, Verrill KC, Trempe JP.** 2006. Effects of adeno-associated virus on adenovirus replication and gene expression during coinfection. J. Virol. **80**:7807–7815.
85. **Tomimatsu N, Mukherjee B, Burma S.** 2009. Distinct roles of ATR and DNA-PKcs in triggering DNA damage responses in ATM-deficient cells. EMBO Rep. **10**:629–635.
86. **Wang S, et al.** 2000. The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. Proc. Natl. Acad. Sci. U. S. A. **97**:1584–1588.
87. **Wang XQ, Redpath JL, Fan ST, Stanbridge EJ.** 2006. ATR dependent activation of Chk2. J. Cell. Physiol. **208**:613–619.
88. **Wang Y, Eckhart W.** 1992. Phosphorylation sites in the amino-terminal region of mouse p53. Proc. Natl. Acad. Sci. U. S. A. **89**:4231–4235.
89. **Warmerdam DO, Kanaar R.** 2010. Dealing with DNA damage: relationships between checkpoint and repair pathways. Mutat. Res. **704**:2–11.
90. **Weindler FW, Heilbronn R.** 1991. A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. J. Virol. **65**:2476–2483.
91. **Weitzman MD, Fisher KJ, Wilson JM.** 1996. Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. J. Virol. **70**:1845–1854.
92. **Weitzman MD, Lilley CE, Chaurushiya MS.** 2010. Genomes in conflict: maintaining genome integrity during virus infection. Annu. Rev. Microbiol. **64**:61–81.
93. **Wilcock D, Lane DP.** 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature **349**:429–431.
94. **Wilkinson DE, Weller SK.** 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. J. Cell Sci. **119**:2695–2703.
95. **Wilkinson DE, Weller SK.** 2005. Inhibition of the herpes simplex virus type 1 DNA polymerase induces hyperphosphorylation of replication protein A and its accumulation at S-phase-specific sites of DNA damage during infection. J. Virol. **79**:7162–7171.
96. **Wilkinson DE, Weller SK.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replica-

tion is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J. Virol. **78**:4783–4796.
97. **Wold MS.** 1997. Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu. Rev. Biochem. **66**:61–92.
98. **Wold MS, Kelly T.** 1988. Purification and characterization of replication protein A, a cellular protein required for in vitro replication of simian virus 40 DNA. Proc. Natl. Acad. Sci. U. S. A. **85**:2523–2527.
99. **Wyman C, Kanaar R.** 2006. DNA double-strand break repair: all's well that ends well. Annu. Rev. Genet. **40**:363–383.
100. **Yang Q, Chen F, Ross J, Trempe JP.** 1995. Inhibition of cellular and SV40 DNA replication by the adeno-associated virus Rep proteins. Virology **207**:246–250.
101. **Yang Q, Chen F, Trempe JP.** 1994. Characterization of cell lines that inducibly express the adeno-associated virus Rep proteins. J. Virol. **68**: 4847–4856.
102. **You JS, Wang M, Lee SH.** 2000. Functional characterization of zinc-finger motif in redox regulation of RPA-ssDNA interaction. Biochemistry **39**:12953–12958.
103. **You Z, Chahwan C, Bailis J, Hunter T, Russell P.** 2005. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. Mol. Cell. Biol. **25**:5363–5379.
104. **Zernik-Kobak M, Vasunia K, Connelly M, Anderson CW, Dixon K.** 1997. Sites of UV-induced phosphorylation of the p34 subunit of replication protein A from HeLa cells. J. Biol. Chem. **272**:23896–23904.
105. **Zhao H, Piwnica-Worms H.** 2001. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol. Cell. Biol. **21**:4129–4139.
106. **Zou L, Elledge SJ.** 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science **300**:1542–1548.
107. **Zou Y, Liu Y, Wu X, Shell SM.** 2006. Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses. J. Cell. Physiol. **208**:267–273.

## 4.2 Further examination of AAV2 and HSV-1 induced DDR

4.2.1 Materials and methods

Cells. DNA-PKcs positive (expressing one copy of DNA-PKcs) and DNA-PKcs negative HCT116 cells were kindly provided by E. Hendrickson (University of Minnesota Medical School, Minneapolis, USA) and maintained in growth medium containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (1% AB). The fibroblast cells AT22 IJE-T yZ5 (expressing ATM) and AT22 IJE-T pEBS7 (ATM-deficient) and GM0166 VA7 (NBS1-deficient) and GM0166 VA7 (expressing NBS1) were kind gifts from Y. Shiloh (Department of Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). These cells were maintained in DMEM supplemented with 10% FBS, 1% AB and 100 µg/ml hygromycin B. HeLa cells were purchased form the American Type Culture Collection (ATCC, Manassas, USA).

Viruses. HSV-1 strain F was kindly provided by B. Roizman (Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, Chicago, USA). wtAAV2 was kindly provided by H. Buening (University of Cologne, Cologne, Germany). rHSV-1vEYFP-ICP4 and rHSV-1 vECFP-ICP4 were kind gifts from R.D. Everett (MRC Virology Unit, University of Glasgow, Glasgow, United Kingdom). Viruses were grown and titrated on Vero cells. Recombinant AAV2mCerryRep (AAVCR) genomes containing the AAV2 ITR flanking the rep ORFs fused at the 5' terminus with the mCherry coding sequence has been described previously (1). Recombinant AAV2YFPRep (AAVYR) genomes contain the AAV2 ITR flanking the Rep ORFs fused at the 5' terminus with the enhanced yellow fluorescence protein (eYFP) coding sequence. See 4.1 for production of recombinant AAV2 (AAVCR and AAVYR).

Plasmids. Plasmids pR68/78 (expressing Rep 78 and Rep 68 from the CMV promoter) and pAAVlacO (containing the AAV2 inverted terminal repeats (ITR) flanking 40 lacO binding sites) were described previously (9, 10, 14). The reporter plasmid pSV2-EYFP/lacI, expressing eYFP fused to the lac repressor (LacI; (34)), was kindly provided by D. L. Spector (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Antibodies. The following primary antibodies were used: Anti-actin (Santa Cruz Biotechnology (SC) 10731; dilution WB 1:10'000), anti-ATM-P-S1981 (Rockland Immunochemicals (RI) 200-301-400; dilution WB 1:500, IF 1:50), anti-HSV-1 ICP8 (Ab 20198; dilution WB 1:1000, IF 1:200), anti-Nbs1 (Novus Biologicals 100-143; dilution WB 1:1000), anti-PARP1 (Sigma, P7605, dilution WB: 1:1000, IF 1:100), anti-AAV2 Rep (Fitzgerald Industries 10R-A111A; dilution WB 1:200), anti-RPA32 (BL A300-244A; dilution WB 1:2000, IF 1:500), anti-RPA32-P-S33 (BL A300-246A; dilution WB: 1:2000, IF 1:1000), anti-RPA32-P-S4/8 (BL A300-245A; dilution WB 1:200, IF 1:200), anti-XRCC1-P-S518/T519/T523 (BL A300-059A-1; dilution WB 1:1000, IF 1:1000), anti-XRCC1 (ab1838 dilution IF 1:200). The following secondary

antibodies were used: Rabbit anti-mouse IgG-horseradish peroxidase (HRP, Sigma A9044; dilution 1:10000), goat anti-rabbit IgG-HRP (Sigma A6154; dilution 1:10000), goat anti-rabbit IgG (H+L)-AF594 (Molecular Probes A11012; dilution 1:1000), goat anti-mouse IgG (H+L)-AF594 (Molecular Probes A11005; dilution 1:1000), goat anti-mouse IgG (H+L)-fluorescein isothiocyanate (FITC, Southern Biotechnology 1031-02; dilution 1:200), goat anti-rabbit IgG (H+L)-FITC (Southern Biotechnology 4050-02; dilution 1:200), goat anti-mouse IgG-Cy5 (Millipore AP181S; dilution 1:500).

Transfection. For immunofluorescence analysis, AT22 IJE-T or HeLa cells (7,5x10^4) were seeded onto cover slips (12 mm ø, Glaswarenfabrik Karl Hecht GmbH&Co KG, Sondheim, Germany) in 24-well plates and transfected with 10ng pR68/78, 25ng pAAVlacO, and 10ng pSV2-EYFP/lacI. Transfection was performed with Lipofectamine® & Plus Reagent (Invitrogen 18324, 11514) according to the manufacturer's protocol. After 3h incubation at 37°C, transfection medium was removed. Cells were washed with optiMEM and infected with wtHSV-1 (MOI 2) for 24h in DMEM (2%FCS, 1%AB).

Western analysis. $10^6$ HCT116 cells or $5x10^6$ AT22 IJE-T cells were seeded into 6cm plates. The following day, the cells were mock-infected, infected with either AAV2 (MOI, 2000) or HSV-1 (MOI 1.5), or coinfected with AAV2 (MOI 2000) and HSV-1 (MOI 1.5) in DMEM supplemented with 2% FBS and 1% AB. After 2h, 8h, 16h, or 24h, cells were prepared for Western analysis as described in (4.1.).

Southern analysis. $9x10^5$ AT22 IJE-T or $2x10^6$ NBS1+/- cells were seeded into 6 cm tissue culture plates. The following day, the cells were mock-infected, infected with wtAAV2 (MOI 10'000) or coinfected with wtAAV2 (MOI 10'000) and HSV-1 (MOI 1) in DMEM supplemented with 2% FBS and 1% AB. After 24h, cells were harvested and extrachromosomal DNA was extracted according to a protocol described by Hirt (15). The DNA was separated on a 1% agarose gel and transferred to nylon membranes (Amersham Hybond-N+ RPN119B). Hybridization with a digoxigenin (DIG)-labeled probe specific for the AAV2 Rep 78-coding sequence and subsequent detection by an anti-DIG antibody conjugated with alkaline phosphatase and activation with the chemiluminescence substrate (CDP Star) was performed according to the manufacturer's protocol (Roche). The DIG-labeled probe was produced with the PCR DIG probe synthesis kit (Roche). PCR amplification was performed from a Rep 78 plasmid as a template. The PCR product was finally purified with the QIAquick PCR purification kit (Qiagen).

ROS experiment. The day before infection, $9x10^5$ AT22 IJE-T cells were seeded into 6 cm tissue culture plates. Cells were coinfected with wtHSV-1 (MOI 1.5) and wtAAV2 (MOI 2000) in the presence or absence of 5mM N-acetylcysteine (NAC). 24h post-infection, cells were subjected to Western analysis (see above) or flow cytometry. For flow cytometry, cells were stained with an indicator for reactive oxygen species (ROS), 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). H2DCFDA is a chemically reduced form of fluorescein. Upon cleavage of the acetate groups by

ROS, the nonfluorescent H2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). 50'000 cells per sample were counted.

Immunofluorescence analysis. AT22 IJE-T cells ($7,5x10^4$) were seeded onto cover slips (12 mm ø, Glaswarenfabrik Karl Hecht GmbH&Co KG, Sondheim, Germany) in 24-well plates. The next day, cells were mock-infected, infected with wtHSV-1 (MOI 1.5), or coinfected with wtHSV-1 (MOI 1.5) and either AAV2CR (MOI 250) or AAV2YR (MOI 250). For fixation and staining procedure see 4.1.

4.2.2 Results

4.2.2.1 RPA32 phosphorylation at S4/8 does not depend on a functional MRN complex.

Although RPA32 was found recruited into both AAV2 and HSV-1 replication compartments, phosphorylation of RPA32 at Ser4/8 was only observed in cells coinfected with AAV2 and HSV-1 (see 4.1). In cells coinfected with Ad and AAV2, phosphorylation of RPA32 at Ser4/8 was also detected and this DDR signaling event was shown to be independent of a functional MRN complex (Mre11, Rad50, NBS1; (28)), because Ad efficiently disrupts the MRN complex in coinfected cells (29) as well as in cells infected with Ad alone (6, 32). In contrast to Ad, HSV-1 does not inactivate the MRN complex but rather induces signaling via ATM, which promotes HSV-1 infection (22). In cells coinfected with AAV2 and HSV-1, there is also no indication for degradation or dislocation of proteins of the MRN complex. Rather, we observed recruitment of at least one of the MRN proteins, NBS1, into AAV2 RCs and its phosphorylation at Ser343 (see 4.1).

Here, we investigated whether phosphorylation of RPA32 at Ser4/8 in cells coinfected with AAV2 and HSV-1 requires a functional MRN complex. In order to abrogate MRN function during AAV2 replication, experiments were performed in cells deficient for NBS1. For visualization of AAV2 DNA replication, we used a visualization system previously developed in our group, employing the interaction of the lac repressor (LacI) fused to eYFP (expressed from plasmid pSV2-EYFP/lacI) with lac operator sequences (LacO) present on a recombinant AAV2 genome (pAAVlacO; (9)). The pAAVlacO DNA contains the AAV2 ITRs flanking 40 lac repressor (LacI) binding sites. Similar to wtAAV2 DNA, due to the presence of the AAV2 ITRs, pAAVlacO serves as substrate for AAV Rep protein and helper virus-mediated DNA replication (9). Immunofluorescence analysis of rAAV2 replication in cells positive or negative for NBS1 revealed that independent of the presence (Fig. 1A) or absence (Fig. 1B) of NBS1, RPA32 is phosphorylated at Ser4/8 and recruited into rAAV2 RCs.

4.2.2.2 Phosphorylation of RPA32 at Ser33 during AAV2 and early HSV-1 replication.

Although, RPA32 was found located in HSV-1 RCs (33, 37, 38), phosphorylation of RPA32 was proposed to not occur upon HSV-1 replication. In line with this, we observed phosphorylation of RPA32 at Ser4/8 only in cells coinfected with AAV2 and HSV-1, but not in cells infected with HSV-1 alone (see 4.1). Surprisingly, when we

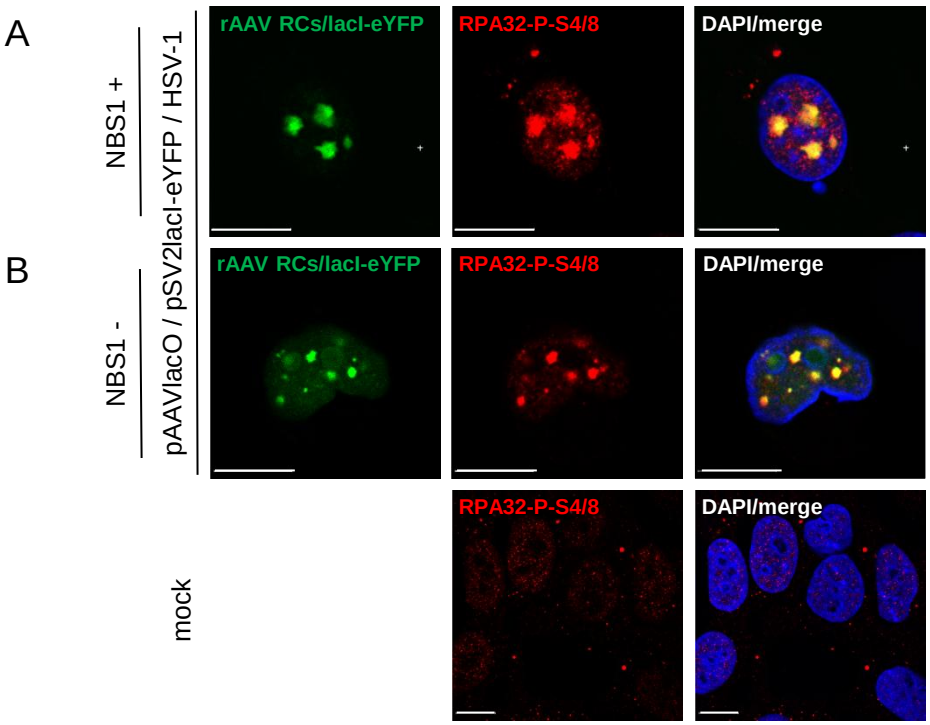



**Fig 1.** RPA32 phosphorylation at S4/8 is independent on a functional MRN complex. **(A and B)** Immunofluorescence analysis of cells, positive (NBS1+, A) or negative (NBS1-, B) for NBS1, transfected with 10ng pR68/78, 25ng pAAVlacO, and 10ng pSV2-EYFP/lacI and infected with wtHSV-1 (MOI 2) for 24h. rAAV RCs (green) were visualized by the binding of LacI-eYFP to LacO-repeats present in the rAAV genome (pAAVlacO). To detect phosphorylated RPA32, cells were stained with an antibody specific for RPA32-P-S4/8 and an AF495-labeled secondary antibody (red). Cellular DNA was stained with DAPI. Scale bar = 5 µm.

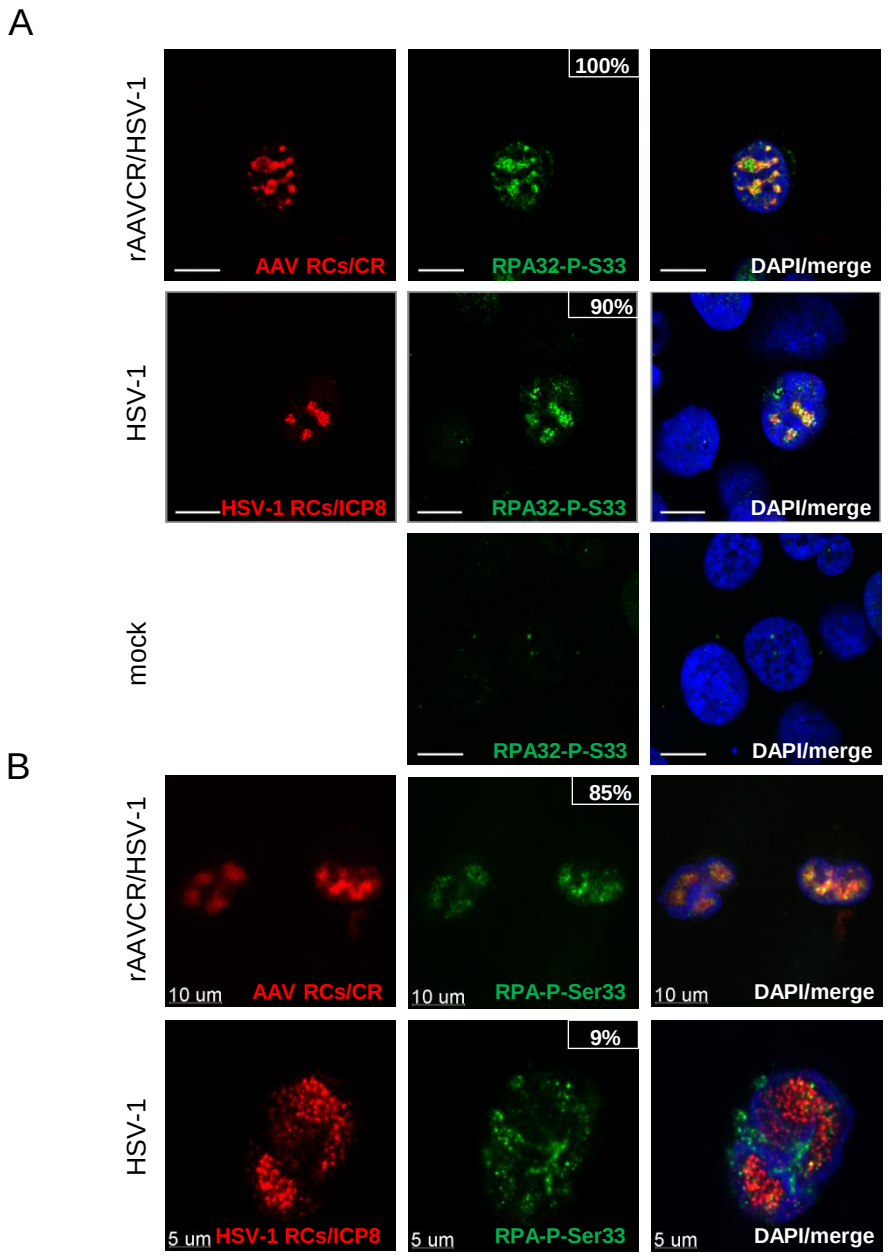



**Fig 2.** Phosphorylation of RPA32 at Ser33 during AAV2 and early HSV-1 replication. **(A and B)** Immunofluorescence analysis of AT22 IJE-T ATM positive cells (yZ5) after infection with wtHSV-1 (MOI 1.5), AAVCR (MOI 250) and wtHSV-1 (MOI 1.5), or mock-infection for 15h (A) or 24h (B). rAAVCR RCs (AAV RCs) were visualized by binding of the rAAVCR-encoded mCherry-Rep68/78 fusion protein (CR) to AAV DNA (red). HSV-1 RCs were visualized with a primary antibody specific for the HSV-1 major DNA binding protein ICP8 and an AF594-labeled secondary antibody (red). To detect phosphorylated RPA32, cells were stained with an antibody against RPA-P-S33 and an FITC-labeled secondary antibody (green). Cellular DNA was stained with DAPI. **(A)** Cell with early AAV2 and HSV-1 RCs. **(B)** Cells with mature AAV2 and HSV-1 RCs late in infection. Scale bar = 10 µm or 5 µm as indicated.

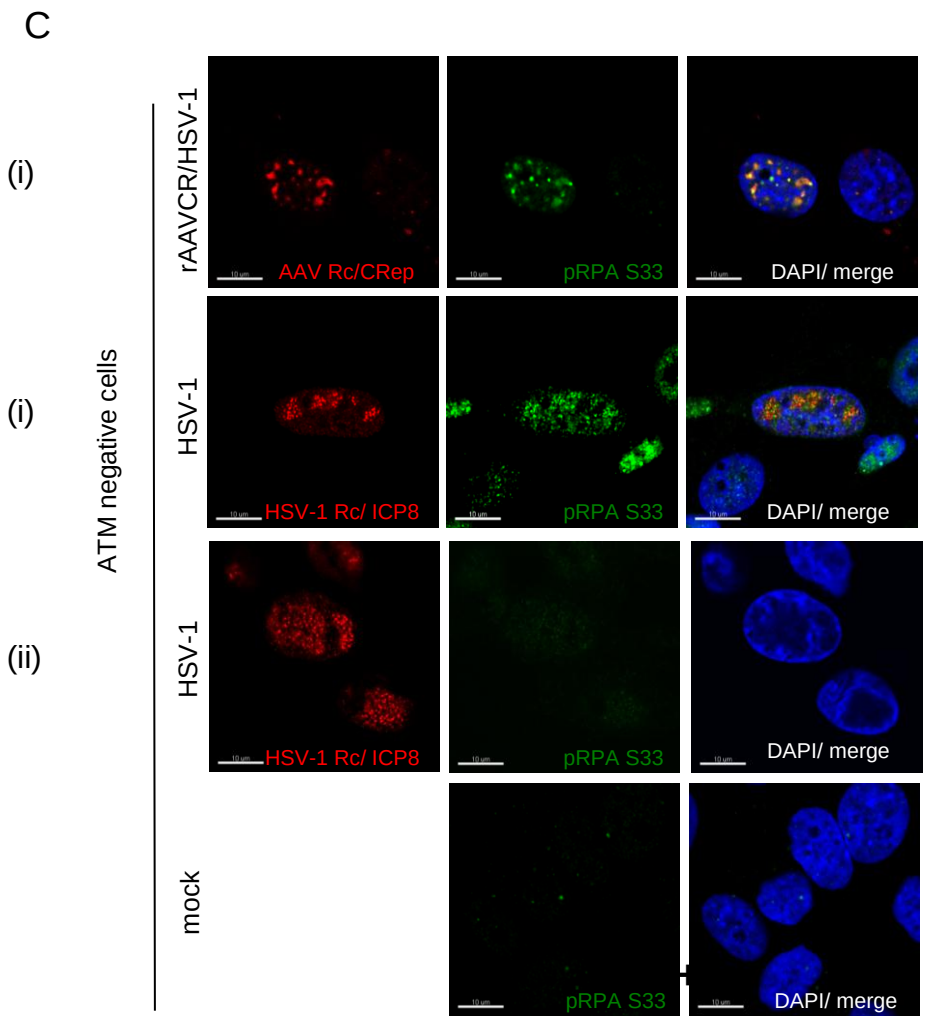



**Fig 2. (C)** Immunofluorescence analysis of AT22 IJE-T pEBS7 (ATM negative) cells after 15h (i) or 24h (ii) infection with HSV-1 (MOI 1.5), rAAVCR (MOI 250) and HSV-1 (MOI 1.5), or mock-infection. rAAVCR and HSV-1 RCs (red) were visualized as described in Fig. 2 A and B. To detect phosphorylated RPA32, cells were stained with an antibody specific for RPA32-P-S33 and an FITC-labeled secondary antibody (green). Cellular DNA was stained with DAPI. Scale bar = 10 µm.

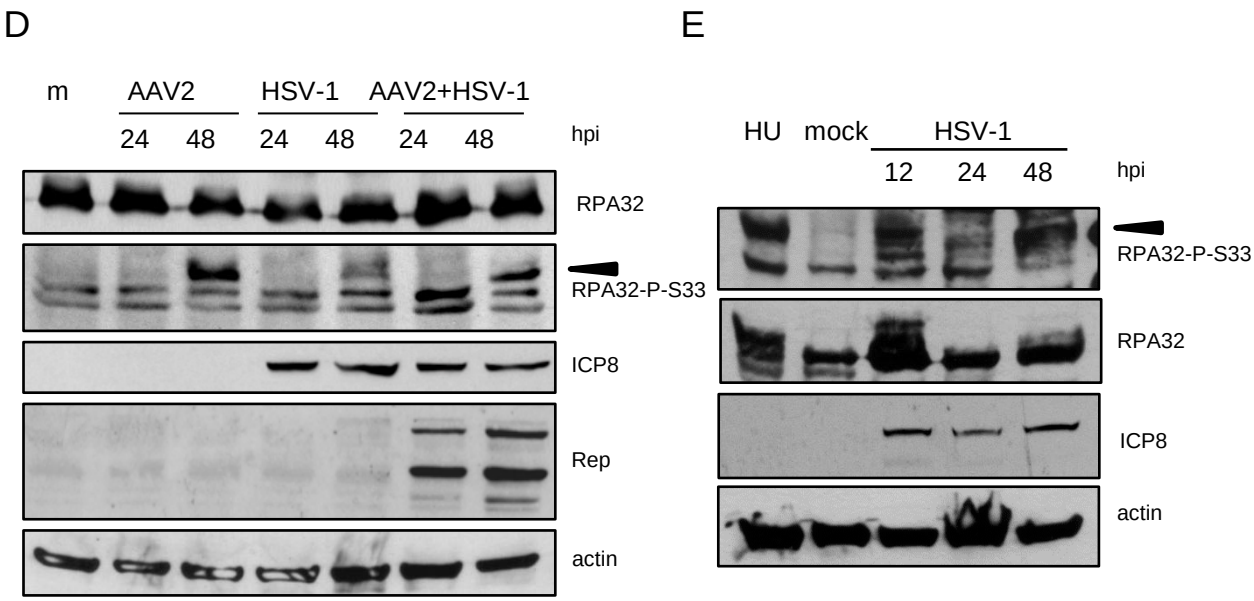



**Fig 2. (D)** Western analysis of HCT116 cells at 24h and 48h after infection with wtAAV (MOI 2000), wtHSV-1 (MOI 1.5), wtAAV (MOI 2000) and wtHSV-1 (MOI 1.5), or mock-infection. Lysates were processed for Western analysis and tested with antibodies against RPA32-P-S33, ICP8, and actin. **(E)** Western analysis of Vero cells at 12h, 24h, and 48h after infection with wtHSV-1 (MOI1.5) or mock-infection. Cells treated with HU (3 mM) served as a RPA32 activation control.

used an antibody specific for RPA32 phosphorylated at Ser33 we did not only detect RPA-P-Ser33 in cells coinfected with AAV2 and HSV-1, but also in cells infected with HSV-1 alone, however with lower intensity (Fig. 2). Immunofluorescence analysis revealed that RPA32-P-S33 colocalized with early AAV2 and HSV-1 RCs in 100% and 90% of the cells, respectively (Fig. 2A). However, late upon infection when viral RCs fill up the entire nucleus, RPA-P-Ser33 colocalization with AAV2 RCs is still observed in most of the compartments (85%), while RPA-P-Ser33 is observed only in few HSV-1 RCs (9%). This data indicates that although AAV2 and HSV-1 can induce phosphorylation of RPA32 at S33, and RPA32-P-S33 is recruited into both AAV2 and HSV-1 RCs, with the onset of HSV-1 infection RPA32-P-Ser33 levels decline and disappear from HSV-1 RCs (Fig. 2B). Similar results were obtained in ATM-deficient cells, in which RPA32-P-Ser33 was found in early and late AAV2 RCs and in early but not in late HSV-1 RCs (Fig. 2C), indicating that ATM is not required for phosphorylation of RPA32 at Ser33 in infected cells. Western analysis of cells harvested at late time points of infection, showed increased levels of RPA32-P-Ser33 when infected with AAV2 or coinfected with AAV2 and HSV-1, but not when infected with HSV-1 alone (Fig. 2D). Interestingly, when Vero cells were infected with HSV-1, RPA32-P-S33 was readily detected at 12h and 48h after infection, while RPA32-P-Ser33 levels were clearly reduced at 24hpi (Fig. 2E). A similar kinetic has been observed also in AT22 cells (Fig. 2B and C). Accumulation of phosphorylated RPA32 especially at 12hpi is also supported by the observed band-shift of total RPA32 (Fig. 2E). As control for the induction of RPA32 phosphorylation, cells were treated with HU (Fig. 2E).

4.2.2.3 Intracellular reactive oxygen species (ROS) levels during AAV2 and HSV-1 replication.

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, and hydrogen peroxides, are produced by the cellular metabolism and have important roles in cell signaling (31). However, ROS levels can increase dramatically under stress conditions, which can directly result in purine, pyrimidine, or deoxyribose modifications (13). In addition, DNA ss breaks (SSBs) and, albeit at a much lower frequency, DNA ds breaks (DSBs), can be induced by ROS (25). DSBs may not only be a result of a direct attack by ROS, but may be also occur when the DNA replication fork encounters unrepaired SSBs or other DNA lesions (25). Given that elevated intracellular ROS can induce DNA damage and even DNA ds breaks, which may further activate a cellular DDR (31), we investigated whether coinfection with AAV2 and HSV-1 modulates the levels of intracellular ROS, and whether ROS influences the activation of a DDR during viral infection. HSV-1 infection has previously been described to increase ROS levels (2, 18, 19, 27). We also observed an increase in ROS levels at 24h after infection of cells with HSV-1 (Fig. 3A). In addition, a similar increase in cellular ROS levels was observed at 24h after coinfected with AAV2 and HSV-1 (Fig. 3A). When using the ROS scavenger N-acetyl cysteine (NAC), HSV-1 infected cells (data not shown) as well as cells coinfected with AAV2 and HSV-1 did not display increased ROS levels at 24hpi (Fig. 3B). However, although ROS levels were decreased to levels similar to those of mock-

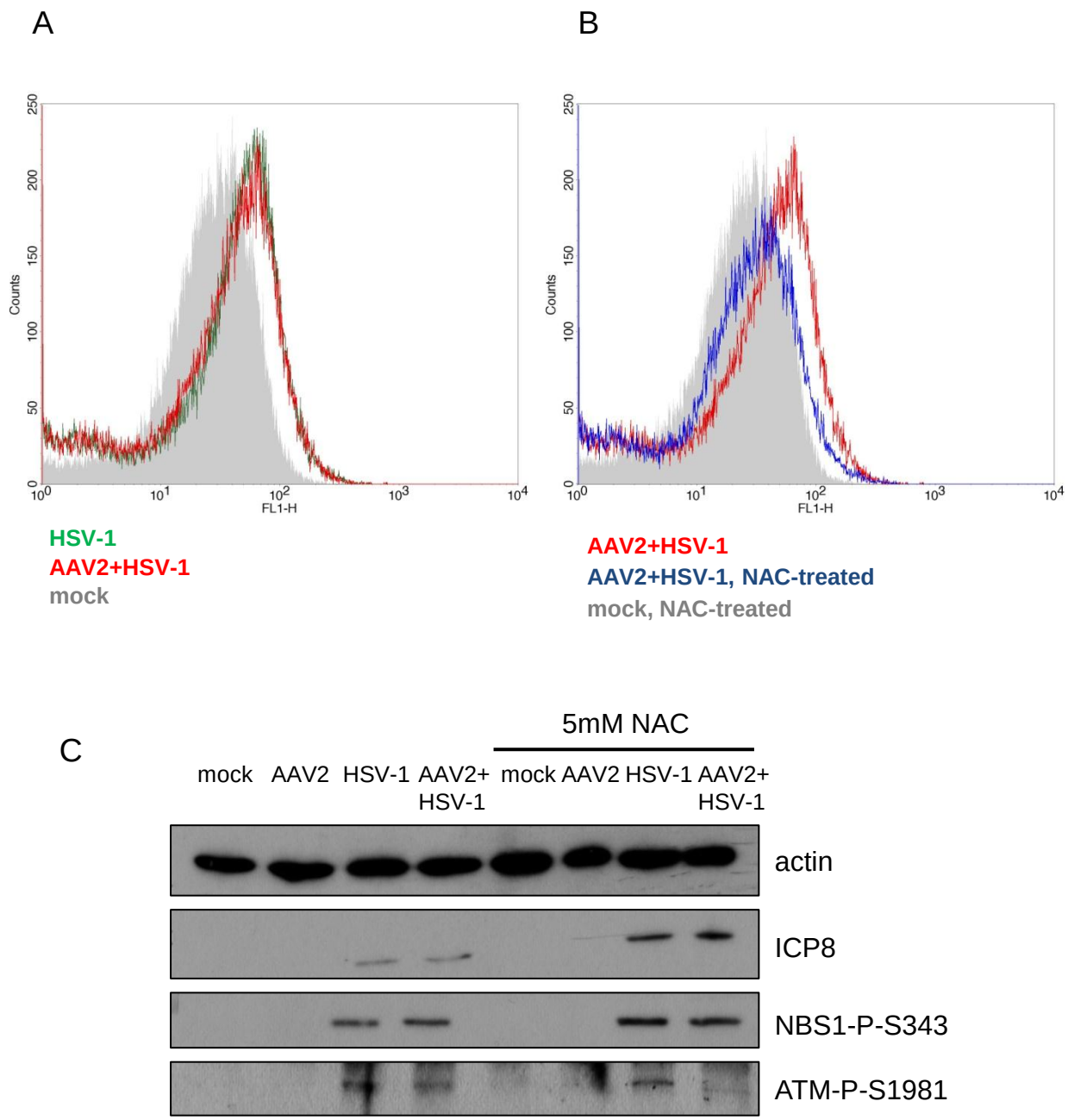



**Fig 3.** Elevated intracellular ROS levels during AAV2 and HSV-1 replication. **(A and B)** AT22 IJE-T yZ5 cells were co-infected with wtHSV-1 (MOI 1.5) and wtAAV (MOI 2000) in the absence (A) or presence of a ROS scavenger (B). Cells were stained with H2DCFDA at 24h after infection, and subjected to flow cytometry. 50'000 cells per sample were counted. **(C)** AT22 IJE-T ATM-positive (yZ5) and ATM negative (pEBS7) cells were mock-infected, infected with either wtAAV (MOI 2000) or wtHSV-1 (MOI 1.5), or co-infected with wtHSV-1 (MOI 1.5) and wtAAV (MOI 2000). After 24h, total protein was isolated and subjected to Western analysis with the indicated antibodies.

infected cells, we did not find a significant decrease in initial DDR signaling via NBS1 and ATM (Fig. 3C).

4.2.2.4 Activation and recruitment of cellular proteins involved in cellular base excision/single-strand break repair (BER/SSBR) upon HSV-1 infection or AAV2 and HSV-1 coinfection.

DNA damage at purine and pyrimidine bases as well as DNA ss breaks can be repaired by the cells base excision/SSB repair (BER/SSBR) machinery (13). BER is typically initiated by DNA glycosylase-mediated removal of the target base and further processing by an endonuclease (APE1), creating a SSB (13). The resulting free termini are processed by polymerase beta and APE1 in order to generate 3′OH and 5′P ends, necessary for proper gap-filling and nick ligation (8). Independent whether gaps result from SSB or BER-intermediates, gap filling and ligation occurs via the same processes (8). It is likely that polymerase beta incorporates the first nucleotide, however further elongation is carried out by replicative DNA polymerases (e.g. polymerase delta; (8)). In addition, RFC is required to load proliferating cell nuclear antigen (PCNA), the sliding clamp of the polymerase, onto the DNA (8). The final ligation step is mediated by the X-ray repair cross complementing 1 (XRCC1)/DNA ligase IIIα complex, however in absence of XRCC1/DNA ligase IIIα, DNA ligase I can also mediate ligation (8). It has been suggested that XRCC1 is recruited to the sites of DNA strand breaks by the multifunctional poly (ADP-ribose) polymerase-1 (PARP-1, also referred to as ADP-ribosyltransferase Diphtheria toxin-like 1 (ARTD1 (16)); (7)).

Because we and others observed elevated ROS upon HSV-1 replication (Fig. 3A; (2, 18, 19, 27)) and AAV2 replication (Fig. 3B), and ROS can result in oxidation of purine or pyrimidine bases as well as SSBs (see above; (13)) we set out to investigate the presence of BER/SSBR factors in HSV-1 and AAV2 RCs. We first assessed expression levels and the localization of PARP1 in infected cells, as PARP1 was shown to be able to recruit the scaffold protein of BER/SSBR, XRCC1, to sites of DNA lesions (7). By immunofluorescence analysis we detected PARP1 within AAV2 and HSV-1 RCs (Fig. 4A). While early upon AAV2 replication, when several small AAV2 RCs are formed within the cell nucleus, abundant PARP1 is located in the entire nucleoplasm (Fig. 4A i), late in AAV2 replication, PARP1 localized exclusively to AAV2 RCs (Fig. 4A ii). Western analysis of cells infected with HSV-1 alone or coinfected with AAV2 and HSV-1 revealed cleavage of PARP1 at late time points of infection, while PARP1 was not cleaved upon infection with AAV2 alone (Fig. 4B). Cleavage of PARP1 in cells infected with HSV-1 was observed previously, and processing of PARP1 was shown to correlate directly with a failure of HSV-1 to prevent activation of apoptotic pathways at late time points of infection (3). The observed decrease of PARP1 levels at 48hpi may be a result of the overall cellular protein shut-off during HSV-1 infection (12) as well as the induction of apoptotic pathways at very late time points (3).

Next, we investigated the localization and activation of XRCC1 upon viral infection. Immunofluorescence analysis revealed that phosphorylated XRCC1 (at S518/T519/T523) as well as total XRCC1 were induced and localized to both AAV2

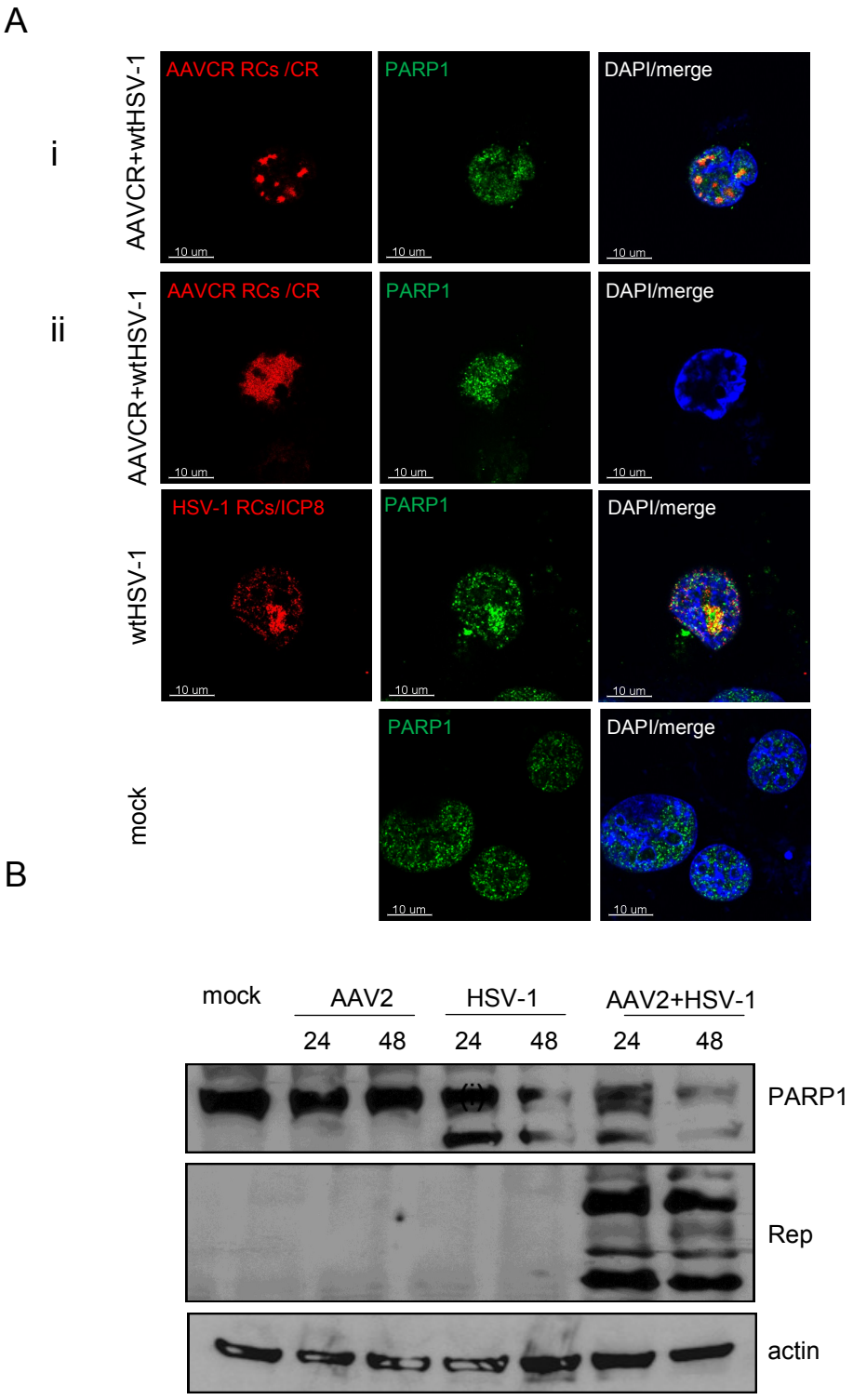



**Fig 4. (A)** Immunofluorescence analysis of AT22 IJE-T cells at 15h (i) and 24h (ii) after infection with wtHSV-1 (MOI 1.5), AAVCR (MOI 500) and wtHSV-1 (MOI 1.5), or mock-infection. AAV2 and HSV-1 RCs (red) were visualized as described in Figure 2. Cells were stained with an antibody against PARP1 and an FITC-labeled secondary antibody (green). Cells with early (i) and mature (ii) AAV2 RCs are shown. Cellular DNA was stained with DAPI. Scale bar = 10 μm. **(B)** Western analysis of HCT116 cells at 24h and 48h after infection with wtAAV (MOI 2000), wtHSV-1 (MOI 1.5), wtAAV (MOI 2000) and wtHSV-1 (MOI 1.5), or mock-infection. Lysates were processed for Western analysis using the indicated antibodies.

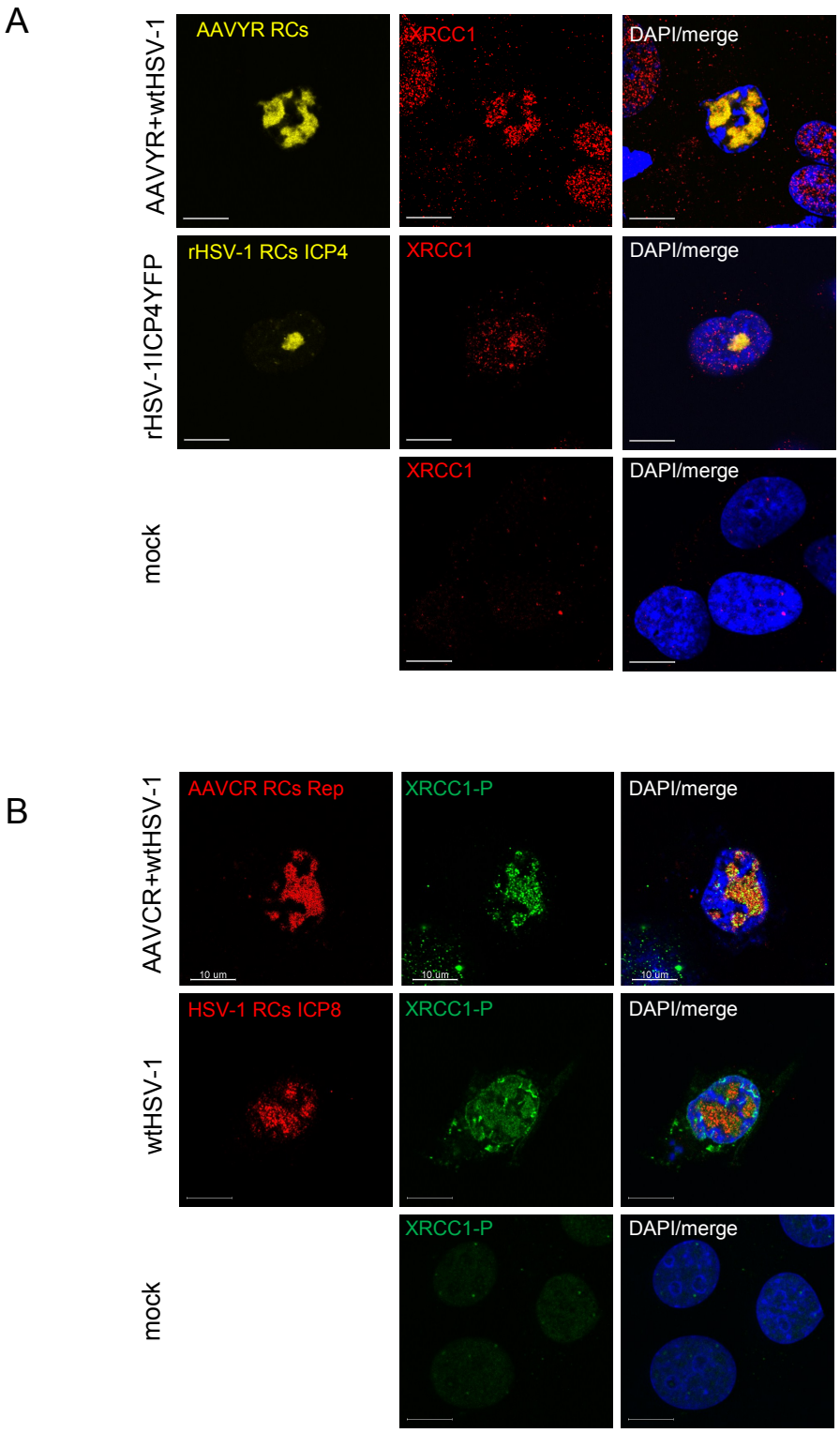
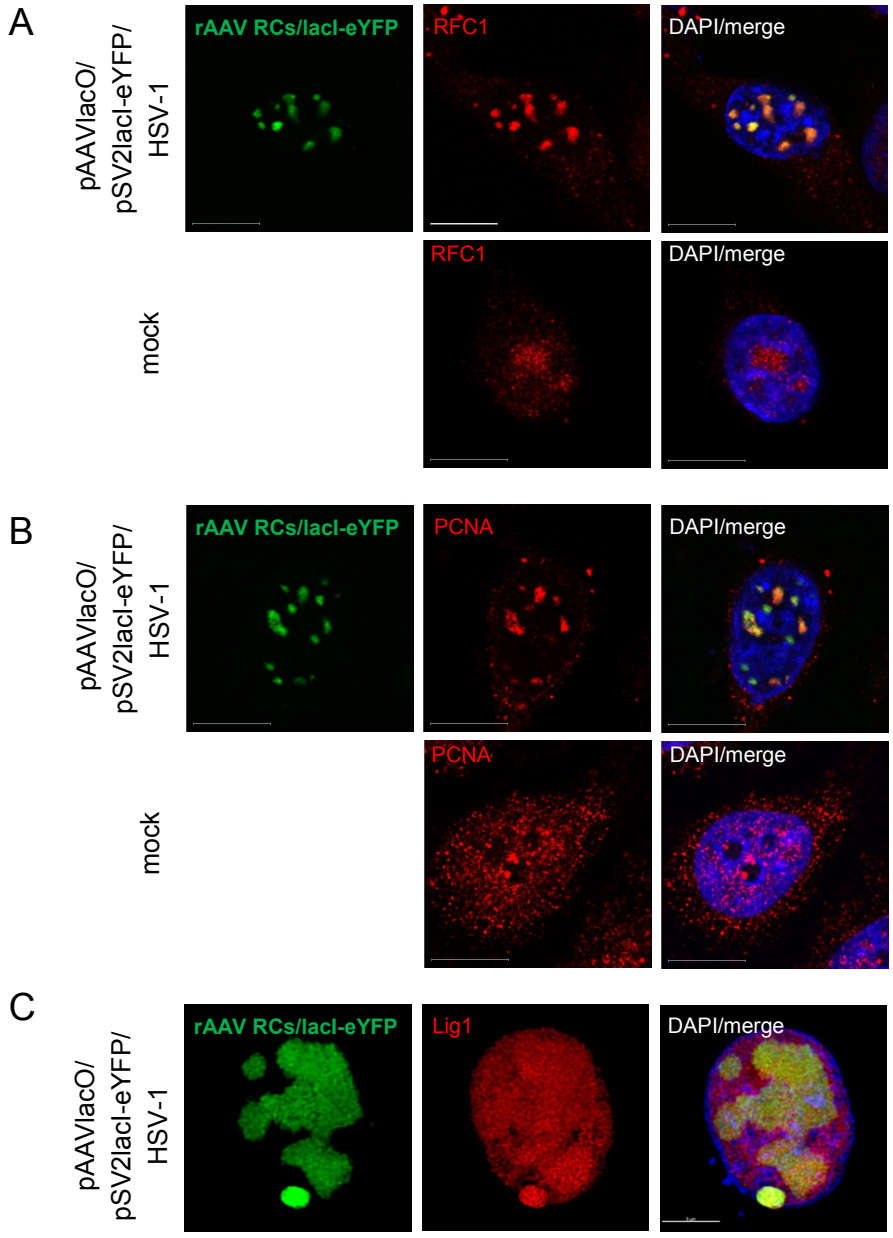



**Fig 5. (A and B)** Immunofluorescence analysis of AT22 IJE-T cells at 24h after infection with rHSV-1ICP4YFP (A)/wtHSV-1(B MOI 1.5), AAVYR (A)/ AAVCR (B, MOI 500) and wtHSV-1 (MOI 1.5), or mock-infection. AAV2 and HSV-1 RCs (red) were visualized as described in Fig. 2. rAAVYR RCs (AAVYR RCs) were visualized by binding of the rAAVYR-encoded eYFP-Rep68/78 fusion protein (YFPRep78/68) to AAV DNA (yellow). Cells were stained with an antibody against XRCC1 (A) or XRCC1-P-S518/T519/T523 (B) and an Alexa-Flour495-labled (A, red) or FITC-labeled (B, green) secondary antibody . Cellular DNA was stained with DAPI. Scale bar = 10 µm.

**Fig 6. (A - C)** Immunofluorescence analysis of HeLa cells transfected with 10ng pR68/78, 25ng pAAVlacO, and 10ng pSV2-EYFP/lacI and infected with wtHSV-1 (MOI 2) for 24h. rAAV2 RCs (green) were visualized by the binding of LacI-YFP to LacO-repeats present in the rAAV genome (pAAVlacO). In addition, cells were stained with an antibody specific for RFC1 (A), PCNA (B), or ligase I (C) and an AF495-labeled secondary antibody (red). Cellular DNA was stained with DAPI. Scale bar = 5 µm



and HSV-1 replication compartments (Fig. 5A and B). In addition to XRCC1, we also detected several other factors of the BER/SSBR machinery located to sites of recombinant AAV2 DNA replication including RFC1, PCNA, and ligase I (Fig 6).

4.2.2.5 The influence of a functional MRN complex on HSV-1 supported AAV2 DNA replication.

As discussed above, NBS1 is a component of the MRN complex (21). There are several reports demonstrating that HSV-1 infection results in the phosphorylation of NBS1 (see 4.1; (22, 30, 38)) and that cells deficient for NBS1 or Mre11 fail to support efficient HSV-1 DNA replication (4, 22). Of note, loss of Mre11 was observed late in HSV-1 infection but it is not clear if this loss has an impact on the efficiency of HSV-1 replication (11). To assess whether the MRN complex influences HSV-1 supported AAV2 infection, AAV2 DNA replication was examined in cells positive or negative for NBS1. In NBS1-positive cells, HSV-1 supported AAV2 replication was much more efficient than in NBS1-negative cells (Fig 7A). We next asked whether reduced AAV2 replication in cells negative for NBS1 is a consequence of a reduced supply of HSV-1 helpervirus functions. HSV-1 helper functions required to induce efficient AAV2 Rep synthesis and AAV2 DNA replication include the HSV-1 IE proteins ICP0, ICP4, and ICP22, as well as the HSV-1 E proteins UL5/UL8/UL52 (helicase-primase complex), ICP8, and UL30/UL42 (DNA polymerase complex; (1)). We investigated the influence of NBS1 on HSV-1 gene expression by Western analysis. While HSV-1 L proteins (VP16 and gC) were significantly reduced in absence of NBS1 (Fig 7B) and also the levels of the E protein ICP8 were noticeably reduced (Fig 7B), levels of the IE protein ICP4 were not affected by the presence or absence of NBS1 (Fig 7B). The levels of other HSV-1 helper proteins in NBS1-deficient cells remain to be determined.

4.2.2.6 The influence of ATM on HSV-1 supported AAV2 DNA replication.

It was previously shown that autophosphorylated ATM is recruited into HSV-1 RCs (see 4.1; (22, 30, 38)) and that cells deficient for ATM fail to support efficient HSV-1 DNA replication (22). Furthermore, it was demonstrated that phosphorylation of NBS1, observed upon HSV-1 replication, requires the ATM kinase (22). As NBS1 turned out to be required for efficient HSV-1 supported AAV2 DNA replication (Fig. 7A), we set out to investigate the role of ATM in this process. Southern analysis of coinfected cells in absence or presence of ATM revealed that, in contrast to HSV-1 DNA replication (22), AAV2 DNA replication is enhanced in absence of ATM (Fig. 8A). Therefore, we next investigated the influence of ATM on HSV-1 gene expression. Western analysis showed that similar to the results observed in NBS1-deficiecnt cells (Fig. 7B), HSV-1 L proteins (VP16 and gC) were significantly reduced in absence of ATM (Fig. 8B) and also the levels of the E protein ICP8 was clearly reduced (Fig. 8B); ICP4 levels were slightly increased at early time points (Fig. 8B).

4.2.3 Discussion

The MRN complex is inhibited in cells infected with Ad alone (6, 32) as well as in cells coinfected with AAV2 and Ad (29). Thus, the observed phosphorylation of

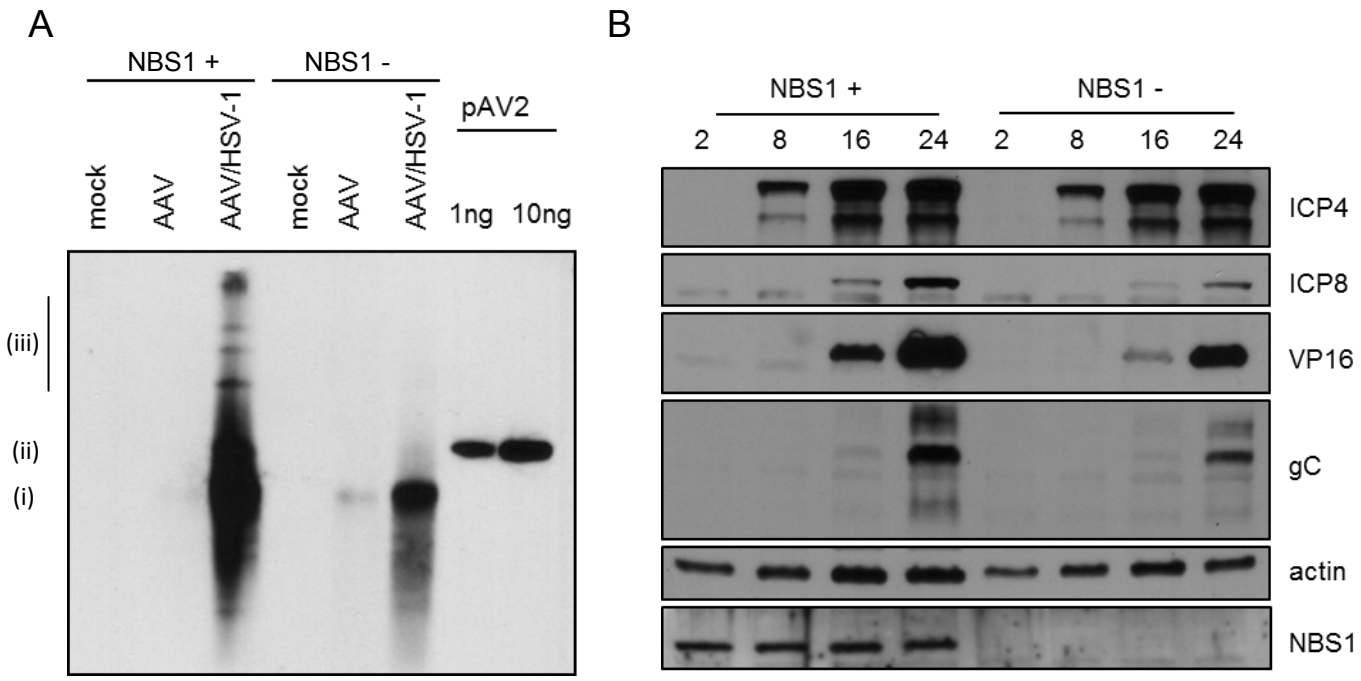



**Fig 7. (A)** Southern analysis of cells positive (NBS+) or negative (NBS-) for NBS1. Cells were mock-infected, infected with wtAAV2 (MOI 10'000) or coinfected with wtAAV2 (MOI 10'000) and wtHSV-1 (MOI 1). After 24h, cells were harvested and processed for Southern analysis. DNA was separated on an agarose gel and transferred to nylon membranes. AAV2 DNA was visualized by hybridization with a DIG-labeled probe specific for AAV2 Rep78 and subsequent detection by an anti-DIG antibody conjugated with alkaline phosphatase. 1ng and 10ng of pAV2 DNA, containing the AAV2 Rep78 sequence, was loaded as a control. (i) AAV2 ssDNA; (ii) AAV2 replication form monomer; (iii) AAV2 replication form dimer/ trimer/tetramer. **(B)** Western analysis of cells positive (NBS+) or negative (NBS-) for NBS1. Cells were mock-infected or infected with HSV-1 (MOI 2). After 2h, 8h, 16h, or 24h, cells were prepared for Western analysis. HSV-1 Immediate early (IE), early (E), and late (L) proteins were detected using antibodies against ICP4, ICP8, VP16, and gC. Actin and NBS1 staining served as controls.

RPA32 at Ser4/8 during AAV2 and Ad coinfection is independent of a functional MRN complex (28). As HSV-1 does not inhibit the MRN-complex (22), it would be possible that MRN is responsible for RPA32 phosphorylation in cells coinfected with AAV2 and HSV-1. However, our data show that neither RPA32 phosphorylation at Ser4/8 nor recruitment into AAV2 RCs depends on a functional MRN complex. Besides phosphorylation of RPA32 at Ser4/8 (see 4.1), we also detected phosphorylation of RPA32 at Ser33 during HSV-1 supported AAV2 replication. Surprisingly, we also observed phosphorylation of RPA32 at Ser33 in cells infected with HSV-1 alone, although phosphorylation of RPA32 at Ser4/8 does not occur in these cells (see 4.1). Interestingly, RPA32-P-Ser33 can only be detected in early but not in late HSV-1 RCs. Because RPA32-P-Ser33 was detected in AAV2 and HSV-1 coinfected ATM negative cells and ATR signaling, seems to be disrupted upon both AAV2 and HSV-1 replication (see 4.1), DNA-PK might be the kinase, responsible for RPA32 phosphorylation at Ser33. However, further investigations are necessary to prove this, in particular RPA32 phosphorylation at Ser33 has to be investigated in cells in which both ATM and ATR are inactivated.

ROS in form of superoxide anions and hydrogen peroxide react readily with proteins, lipids, carbohydrates, and nucleic acids (5). ROS are produced by a large number of enzymes (xanthine oxidase, nitric oxide synthetase, p450 cytochromes) and organelles (mitochondria, peroxisomes) as a metabolic byproduct; however, elevated ROS levels are produced by NADPH oxidases (NOX; (5)). Given that infection of cells with HSV-1 was shown to increase ROS levels (2, 18, 19, 27) and ROS can induce DNA damage (31), we investigated whether coinfection with AAV2 and HSV-1 also influences intracellular ROS levels and whether this is linked to the observed activation of DDR signaling during viral infection. Although we observed elevated ROS levels during both HSV-1 and AAV2 replication, by infecting cells in presence of the ROS scavenger NAC, we did not observe a significant inhibition of two different central components of DDR signaling, NBS1 and ATM. Therefore, we conclude that viral DNA and/or viral proteins are sufficient to activate DDR signaling. Still elevated ROS levels in infected cells may affect viral DNA integrity.

The most common types of DNA damage induced by ROS include oxidation of purine or pyrimidine bases and DNA SSBs (13) which can be repaired by the cellular BER/SSBR machinery. We therefore set out to investigate the presence of BER/SSBR factors in HSV-1 and AAV2 RCs. PARP1 and phosphorylated XRCC1 were found in both, AAV2 and HSV-1 RCs. Similar to its role in cellular BER/SSBR (7), PARP1 might mediate recruitment of XRCC1 into viral replication compartments. We also detected several other proteins involved in cellular BER/SSBR, including RCF1, PCNA, and ligase I in AAV2 RCs. Since HSV-1 infection alone (2, 18, 19, 27) as well as coinfection with AAV2 and HSV-1 resulted in increased ROS levels, and ROS is able to induce oxidized bases and single-strand breaks (SSBs), it is possible that BER/SSBR proteins protect viral DNA from ROS induced DNA damage. During AAV2 replication, the BER/SSBR machinery might also be involved in terminal resolution of the ITR after Rep 78 induced nicking at the TRS. Further experiments are needed to test this hypothesis. It has to be mentioned that although most studies have focused on the role of PARP1 in DNA damage sensing and repair pathways

there is increasing evidence that PARP1 also plays a central role in gene regulation and chromatin modulation (20). In further experiments it would be very interesting to examine a potential influence of PARP1 on viral gene regulation as well as modification of histones associated with AAV2 DNA (24) in infected cells.

In a last set of experiments, we investigated the role of NBS1 and ATM in HSV-1 supported AAV2 DNA replication. Experiments in NBS1 negative cells indicated that the presence of a functional MRN complex or NBS1 clearly aids HSV-1 supported AAV2 DNA replication. Surprisingly, and in contrast to the results observed in cells negative for NBS1, HSV-1 supported AAV2 DNA replication was shown to be increased in ATM-deficient cells. These data indicate that although ATM signaling seems to be necessary for efficient HSV-1 DNA replication (22), the presence of the ATM kinase is a limiting factor for HSV-1 supported AAV2 DNA replication. Further experiments are necessary to investigate whether alterations in expression levels of HSV-1 IE and E genes in NBS1 and ATM negative cells also occur in coinfected cells and whether this is linked to the efficiency of AAV2 DNA replication.

## 2.2.4 References (chapters 4.2)


1. **Alazard-Dany, N., A. Nicolas, A. Ploquin, R. Strasser, A. Greco, A. L. Epstein, C. Fraefel, A. Salvetti, and P. O'Hare.** 2009. Definition of Herpes Simplex Virus Type 1 Helper Activities for Adeno-Associated Virus Early Replication Events. PLoS Pathog **5**:e1000340.
2. **Aubert, M., Z. Chen, R. Lang, C. H. Dang, C. Fowler, D. D. Sloan, and K. R. Jerome.** 2008. The antiapoptotic herpes simplex virus glycoprotein J localizes to multiple cellular organelles and induces reactive oxygen species formation. J Virol **82**:617–629.
3. **Aubert, M., L. E. Pomeranz, and J. A. Blaho.** 2007. Herpes simplex virus blocks apoptosis by precluding mitochondrial cytochrome c release independent of caspase activation in infected human epithelial cells. Apoptosis **12**:19–35.
4. **Balasubramanian, N., P. Bai, G. Buchek, G. Korza, and S. K. Weller.** 2010. Physical Interaction between the Herpes Simplex Virus Type 1 Exonuclease, UL12, and the DNA Double-Strand Break-Sensing MRN Complex. J Virol **84**:12504–12514.
5. **Brieger, K., S. Schiavone, F. J. Miller, JR, and K.-H. Krause.** 2012. Reactive oxygen species: from health to disease. Swiss Med Wkly **142**:w13659.
6. **Carson, C., R. Schwartz, T. Stracker, C. Lilley, D. Lee, and M. Weitzman.** 2003. The Mre11 complex is required for ATM activation and the G2/M checkpoint. The EMBO journal **22**:6610–6620.
7. **El-Khamisy, S. F.** 2003. A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage. Nucleic Acids Research **31**:5526–5533.
8. **Fortini, P., and E. Dogliotti.** 2007. Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways. DNA Repair (Amst) **6**:398–409.
9. **Fraefel, C., A. G. Bittermann, H. Bueler, I. Heid, T. Bachi, and M. Ackermann.** 2004. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. J Virol **78**:389–398.
10. **Glauser, D. L., O. Saydam, N. A. Balsiger, I. Heid, R. M. Linden, M. Ackermann, and C. Fraefel.** 2005. Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. J Virol **79**:12218–12230.
11. **Gregory, D. A., and S. L. Bachenheimer.** 2008. Characterization of mre11 loss following HSV-1 infection. Virology **373**:124–136.
12. **Hardwicke, m., and Sandri-Goldin r.m.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J Virol **68**:4797–4810.
13. **Hegde, M. L., A. K. Mantha, T. K. Hazra, K. K. Bhakat, S. Mitra, and B. Szczesny.** 2012. Oxidative genome damage and its repair: Implications in aging and neurodegenerative diseases. Mechanisms of Ageing and Development **133**:157–168.
14. **Heister, T., I. Heid, M. Ackermann, and C. Fraefel.** 2002. Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. J Virol **76**:7163–7173.
15. **Hirt, B.** 1969. Replicating molecules of polyoma virus DNA. J Mol Biol **40**:141–144.
16. **Hottiger, M. O., P. O. Hassa, B. Lüscher, H. Schüler, and F. Koch-Nolte.** 2010. Toward a unified nomenclature for mammalian ADP-ribosyltransferases. Trends in Biochemical Sciences **35**:208–219.
17. **Jiang, M., and M. J. Imperiale.** 2012. Design stars: how small DNA viruses remodel the host nucleus. Future Virology **7**:445–459.
18. **Kavouras, J. H., E. Prandovszky, K. Valyi-Nagy, S. K. Kovacs, V. Tiwari, M. Kovacs, D. Shukla, and T. Valyi-Nagy.** 2007. Herpes simplex virus type 1 infection induces oxidative stress and the release of bioactive lipid peroxidation by-products in mouse P19N neural cell cultures. Journal of neurovirology **13**:416–425.
19. **Kim, J. C., S. H. Choi, J. K. Kim, Y. Kim, H. J. Kim, J. S. Im, S. Y. Lee, J. M. Choi, H. M. Lee, and J. K. Ahn.** 2008. [Herpes simplex virus type 1 ICP27 induces apoptotic cell

death by increasing intracellular reactive oxygen species]. Molekuliarnaia biologiia **42**:470–477.
20. **Kraus, W. L., and M. O. Hottiger.** 2013. PARP-1 and gene regulation: Progress and puzzles. Molecular Aspects of Medicine.
21. **Lavin, M. F., and S. Kozlov.** 2007. ATM activation and DNA damage response. Cell Cycle **6**:931–942.
22. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A **102**:5844–5849.
23. **Lilley, C. E., R. A. Schwartz, and M. D. Weitzman.** 2007. Using or abusing: viruses and the cellular DNA damage response. Trends in Microbiology **15**:119–126.
24. **Marcus-Sekura, C. J., and B. J. Carter.** 1983. Chromatin-like structure of adeno-associated virus DNA in infected cells. J Virol **48**:79–87.
25. **McKinnon, P. J., and K. W. Caldecott.** 2007. DNA Strand Break Repair and Human Genetic Disease. Annu. Rev. Genom. Human Genet. **8**:37–55.
26. **Nikitin, P. A., and M. A. Luftig.** 2011. At a crossroads: human DNA tumor viruses and the host DNA damage response. Future Virology **6**:813–830.
27. **Schachtele, S. J., S. Hu, M. R. Little, and J. R. Lokensgard.** 2010. Herpes simplex virus induces neural oxidative damage via microglial cell Toll-like receptor-2. Journal of neuroinflammation **7**:35.
28. **Schwartz, R. A., C. T. Carson, C. Schuberth, and M. D. Weitzman.** 2009. Adeno-Associated Virus Replication Induces a DNA Damage Response Coordinated by DNA-Dependent Protein Kinase. J Virol **83**:6269–6278.
29. **Schwartz, R. A., J. A. Palacios, G. D. Cassell, S. Adam, M. Giacca, and M. D. Weitzman.** 2007. The Mre11/Rad50/Nbs1 Complex Limits Adeno-Associated Virus Transduction and Replication. J Virol **81**:12936–12945.
30. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. The Journal of biological chemistry **280**:30336–30341.
31. **Shrivastav, M., L. P. de Haro, and J. A. Nickoloff.** 2008. Regulation of DNA double-strand break repair pathway choice. Cell Res **18**:134–147.
32. **Stracker, T. H., C. T. Carson, and M. D. Weitzman.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature **418**:348–352.
33. **Taylor, T. J., and D. M. Knipe.** 2004. Proteomics of herpes simplex virus replication compartments. association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J Virol **78**:5856–5866.
34. **Tsukamoto, T., N. Hashiguchi, S. M. Janicki, T. Tumbar, A. S. Belmont, and D. L. Spector.** 2000. Visualization of gene activity in living cells. Nature cell biology **2**:871–878.
35. **Turnell, A. S., and R. J. Grand.** 2012. DNA viruses and the cellular DNA-damage response. Journal of General Virology **93**:2076–2097.
36. **Weitzman, M. D., C. T. Carson, R. A. Schwartz, and C. E. Lilley.** 2004. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) **3**:1165–1173.
37. **Wilcock, D., and D. P. Lane.** 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature **349**:429–431.
38. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of virology **78**:4783–4796.

33. **Taylor, T. J., and D. M. Knipe.** 2004. Proteomics of herpes simplex virus replication compartments. association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J Virol **78**:5856–5866.
34. **Tsukamoto, T., N. Hashiguchi, S. M. Janicki, T. Tumbar, A. S. Belmont, and D. L. Spector.** 2000. Visualization of gene activity in living cells. Nature cell biology **2**:871–878.
35. **Turnell, A. S., and R. J. Grand.** 2012. DNA viruses and the cellular DNA-damage response. Journal of General Virology **93**:2076–2097.
36. **Weitzman, M. D., C. T. Carson, R. A. Schwartz, and C. E. Lilley.** 2004. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) **3**:1165–1173.
37. **Wilcock, D., and D. P. Lane.** 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature **349**:429–431.
38. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of virology **78**:4783–4796.

## 4.3 Investigation of the AAV2 mediated interference with HSV-1-ICP0-induced degradation of DNA-PKcs.

Background and aim of the project.
Immunofluorescence analysis, flow cytometry, as well as fluorescence-activated cell sorting (FACS) and subsequent Western analysis of cells infected with AAV2 and/or HSV-1 showed that HSV-1 induced degradation of DNA-PKcs is delayed in coinfected cells. In addition, we observed phosphorylation of DNA-PKcs at S2056, which is necessary for its kinase activity, only in cells coinfected with AAV2 and HSV-1 but not in cells infected with HSV-1 alone (see 4.1). To explore potential mechanisms responsible for the delayed degradation of DNA-PKcs in coinfected cells, we first assessed the levels of HSV-1 ICP0 and USP7, another cellular target of ICP0-mediated degradation. Corroborating previous published work (6), we did not detect reduced levels of ICP0 in coinfected cells, when compared to cells infected with HSV-1 alone. Moreover, we excluded the possibility of an overall inactivation of ICP0-mediated cellular protein-degradation, as USP7 levels were comparable in cells infected with HSV-1 alone and in coinfected cells (see 4.1).
Therefore, we investigated the question how AAV2 interferes with HSV-1 induced degradation of DNA-PKcs?

4.2.1 Materials and methods.
Cells. The fibroblast cells AT22 IJE-T yZ5 (expressing ATM) and AT22 IJE-T pEBS7 (lacking ATM) were kind gifts from Y. Shiloh (Department of Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1% AB and 100 µg/ml hygromycin B. Cells were maintained at 37°C in a 95% air/5% CO2 atmosphere.

Viruses. HSV-1 strain F as well as the HSV-1 ICP0 deletion mutant, rHSV-1 R7914, carrying an aspartate-to-alanine substitution in the ICP0 coding sequence at amino acid position D199 was kindly provided by B. Roizman (Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, Chicago, USA). rHSV-1eCFP-ICP4 has been described in 4.2. The recombinant HSV-1, rHSV-1 D8, which lacks the nuclear localization signal in the ICP0 coding sequences and therefore expresses cytosol restricted ICP0, was a kind gift R.D. Everett (MRC Virology Unit, University of Glasgow, Glasgow, United Kingdom). Viruses were grown and titrated in Vero cells. AAV2 and Ad2 were kindly provided by H. Buening (University of Cologne, Cologne, Germany) and U. Greber (University of Zurich, Zurich, Switzerland), respectively. Recombinant AAV2 AAV2CR containing the AAV2 ITR flanking the *rep* ORF fused at the 5' terminus with mCherry has been described previously (1). In addition, a recombinant AAV2eYFPRep (AAVYR), containing the AAV2 ITR flanking the *rep* ORF fused at the 5' terminus with the eYFP coding sequence was cloned by replacing the mCherry sequence in the pAAVCR (1) with the eYFP sequence. Recombinant AAVCR and AAVYR particles of AAV2 were produced by transient transfection of 293 cells with an Ad helper plasmid purified on two successive CsCl

gradients, and genome containing particles were titered by qPCR. rAAV2GFP, containing the AAV2 ITRs flanking the eGFP coding sequence under control of the HCMV IE1 enhancer/promoter, was kindly provided by M. Linden (King's College London School of Medicine, London, UK).

Plasmids. Plasmids pNLS(GFP), pRep52(GFP) and pRep78(GFP), which encode the eGFP coding sequence fused with a nuclear localization signal (NLS), Rep 52 or 78, respectively, under control of the HCMV IE1 enhancer/promoter were described previously (5). Plasmid pICP0, containing the HSV-1 ICP0 coding sequence under control of the HCMV IE1 enhancer/promoter, was kindly provided by P. F. O'Hare (Department of Medicine, Imperial College, London, UK).

siRNAs. siRNA against three different Ku70 target sequences were used in Ku70-knock down experiments: Ku70-1 5′-UUCAGGUGACUCCUCCAGGTT-3′, Ku70-2 5′ UUCUCUUGGUAACUUUCCCTT-3′ and Ku70-3 5′-GAUGCCCUUUACUGAAAAATT-3′. Ku70 siRNAs were ordered from Microsynth AG (Balgach, Switzerland). Scrambled siRNA (Control siRNA-A, sc-37007) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

Antibodies. The following primary antibodies were used: Anti-actin (Santa Cruz Biotechnology (SC) 10731; dilution WB 1:10'000), anti-DNA-PKcs (Ab 32566; dilution WB 1:500, IF 1:50, Flow cytometry 1:250), anti-DNA-PKcs-P-S2056 (Ab 18192; dilution WB 1:2000, IF 1:500), anti-HSV-1 ICP8 (Ab 20198; dilution WB 1:1000, IF 1:200), anti-Ku70 (NeoMarkers; dilution WB 1:5000), anti-HSV-1 ICP0 (Ab 6513; dilution WB 1:8000, IF 1:2500), anti-AAV2 Rep (Fitzgerald Industries 10R-A111A; dilution WB 1:200). The following secondary antibodies were used: Rabbit anti-mouse IgG-horseradish peroxidase (HRP, Sigma A9044; dilution 1:10000), goat anti-rabbit IgG-HRP (Sigma A6154; dilution 1:10000), goat anti-rabbit IgG (H+L)-AlexaFlour(AF)405 (Molecular Probes A31556; dilution 1:500), goat anti-rabbit IgG (H+L)-AF594 (Molecular Probes A11012; dilution 1:1000), goat anti-mouse IgG (H+L)-AF594 (Molecular Probes A11005; dilution 1:1000), goat anti-mouse IgG (H+L)-fluorescein isothiocyanate (FITC, Southern Biotechnology 1031-02; dilution 1:200), goat anti-rabbit IgG (H+L)-FITC (Southern Biotechnology 4050-02; dilution 1:200)

Transfection and infection. AT22 IJE-T cells, seeded the day before transfection, were transfected with Lipofectamine® LTX and Plus Reagent (Invitrogen 15338030) according to the manufacturer's protocol. Transfections were carried out in two different plate formats: 12-well plates (Western analysis) and 24-well plates (immunofluorescence analysis). For Western analysis, $2x10^5$ AT22 IJE-T cells were transfected with 0.5 µg of the individual pRepGFP plasmids or the control plasmid pNLSGFP. After 3h incubation at 37°C and 5 % $CO_2$, transfection medium was removed, cells were washed with OptiMEM and mock-infected or infected with wtHSV-1 (MOI 3) for 24h in DMEM (2%FCS, 1%AB). For immunofluorescence analysis, AT22 IJE-T cells ($7,5x10^4$) were seeded onto cover slips (12 mm ø,

Glaswarenfabrik Karl Hecht GmbH&Co KG, Sondheim, Germany) in 24-well plates and transfected with 0.25 µg of pRepGFP and 0.1ug of pICP0. After 3h incubation at 37°C, transfection medium was removed, cells were washed with optiMEM and incubated for 24h in DMEM containing 2%FCS and 1%AB.

siRNA transfections and infections. One day before siRNA transfection, AT22 IJE-T cells ($7{,}5x10^4$) were seeded onto cover slips (12 mm ø, Glaswarenfabrik Karl Hecht GmbH&Co KG, Sondheim, Germany) in 24-well plates. Ku70 siRNA (20µM of each) or scrambled siRNA transfection was performed with Lipofectamine® LTX & Plus Reagent (Invitrogen 15338030) according to the according to the manufacturer's protocol. After 4h, the transfection medium was removed, and cells were incubated for 48h in DMEM containing 2%FCS and 1%AB. Subsequently, cells were coinfected with AAVCR (MOI 2000) and HSV-1 (MOI 3) in DMEM containing 2%FCS and 1%AB for 24h and then subjected to immunofluorescence analysis.

Western analysis. See 4.1

Fluorescence-activated cell sorting (FACS) and subsequent Western analysis. $6.6x10^6$ AT22IJE-T cells were seeded into 10 cm cell culture dishes in DMEM 10%FCS, 1%AB. The next day, cells were mock-infected, coinfected with wtHSV-1 (MOI 3) and rAAVGFP (MOI 4000), or coinfected with wtHSV-1 (MOI 3), rAAVGFP (MOI 2000), and wtAAV2 (MOI 2000). Equal numbers of eGFP-positive cells were sorted and prepared for Western analysis as described in 4.1. The same number of mock-infected cells was used as control. FACS and Western analysis of AT22IJE-T cells infected with rHSV-1eCFP-ICP4 or coinfected with rHSV-1eCFP-ICP4 and AAVCR was performed as described in 4.1.

Immunofluorescence analysis. AT22 IJE-T cells ($7{,}5x10^4$) in DMEM 10%FCS, 1%AB were seeded onto cover slips (12 mm ø, Glaswarenfabrik Karl Hecht GmbH&Co KG, Sondheim, Germany) in 24-well plates. The next day, cells were mock-infected, infected with wtHSV-1 (MOI 3), or rHSV-1D8 (MOI 3), or rHSV-1 R7914 (MOI 3), or coinfected with rAAV2CR (MOI 500) and either wtHSV-1 (MOI 3), or rHSV-1D8 (MOI 3) or rHSV-1 R7914 (MOI 3). For fixation and staining procedure see 4.1).

4.3.2 Results

4.3.2.1 Influence of ICP0 localization on degradation of DNA-PKcs in coinfected cells. We confirmed that ICP0 localized both in the nucleus and in the cytoplasm of HSV-1 infected cells (Fig. 1A), as has previously been reported (7, 8). It has been shown that the localization of HSV-1 ICP0 in infected cells depends on the progress of HSV-1 infection (7, 8). Within several hours after infection, ICP0 translocates form the nucleus to the cytoplasm (7). This translocation was dependent on a function expressed late in infection (8) and could be blocked by treatment of the cells with the proteasome inhibitor MG132 (10). Interestingly, translocation of ICP0 is not only dependent on the progress of infection but also on the cell type. In Vero cells for

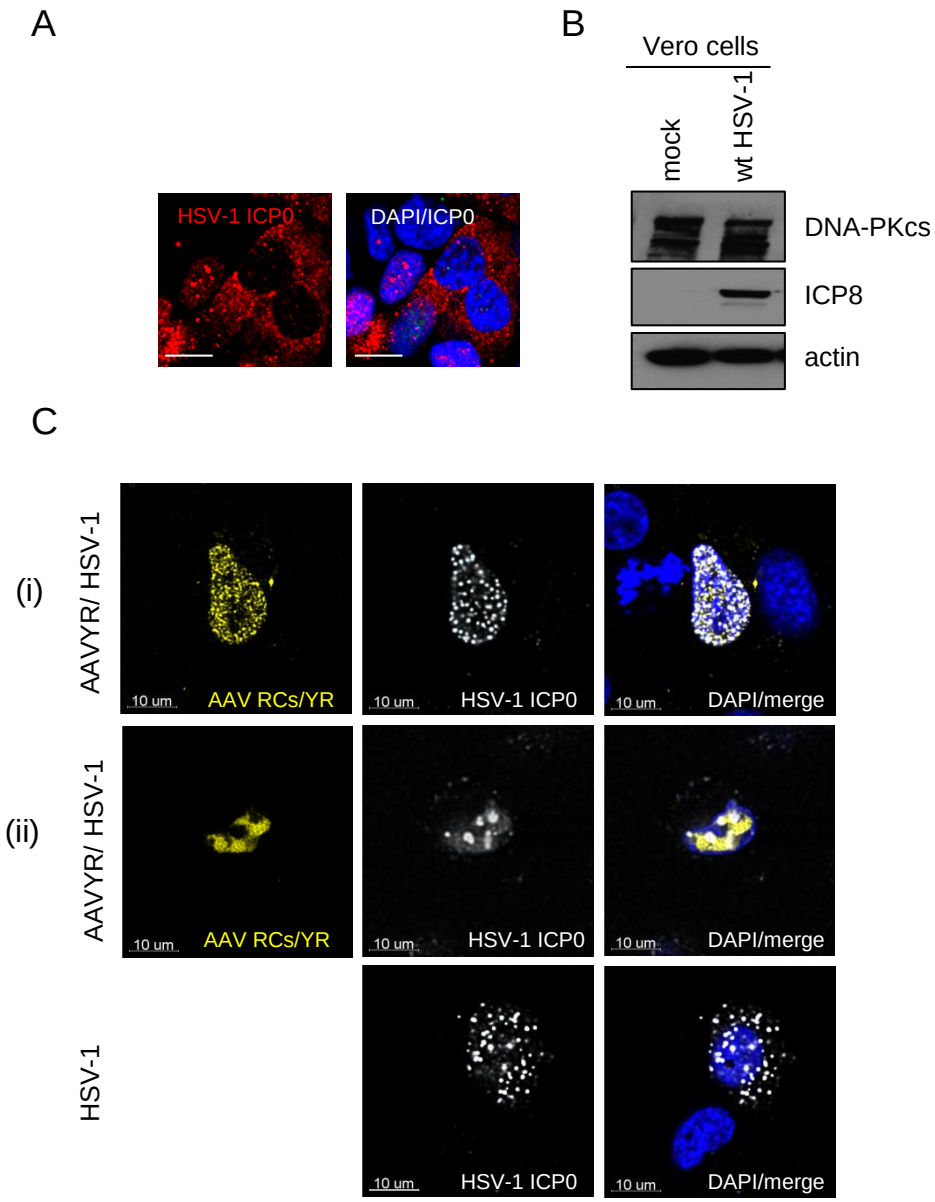



**Fig 1**. ICP0 localization in infected cells and degradation of DNA-PKcs. **(A and C)** Immunofluorescence analysis of AT22IJE-T cells infected with wtHSV-1 (MOI 1.5) for 24h or AAVYR (MOI 500) and wtHSV-1 (MOI 1.5, C) for 15h (i) or 24hp (ii). AAVYR replication compartments (AAV RCs, C) were visualized by binding of the AAVYR-encoded eYFP-Rep 68/78 fusion protein (YR) to AAV DNA (yellow). Cells were stained with an antibody specific for ICP0 and AF 594-labeled (red, A) or FITC-labeled (white, C) secondary antibody. DAPI was used to stain cell nuclei. Scale bars, 20 μm (A) or 10 μm (C). **(B)** Western analysis of Vero cells at 24h after infection with wtHSV-1 (MOI 1.5) or mock-infection. Lysates were processed for Western analysis and stained with antibodies against DNA-PKcs, ICP8, and actin.

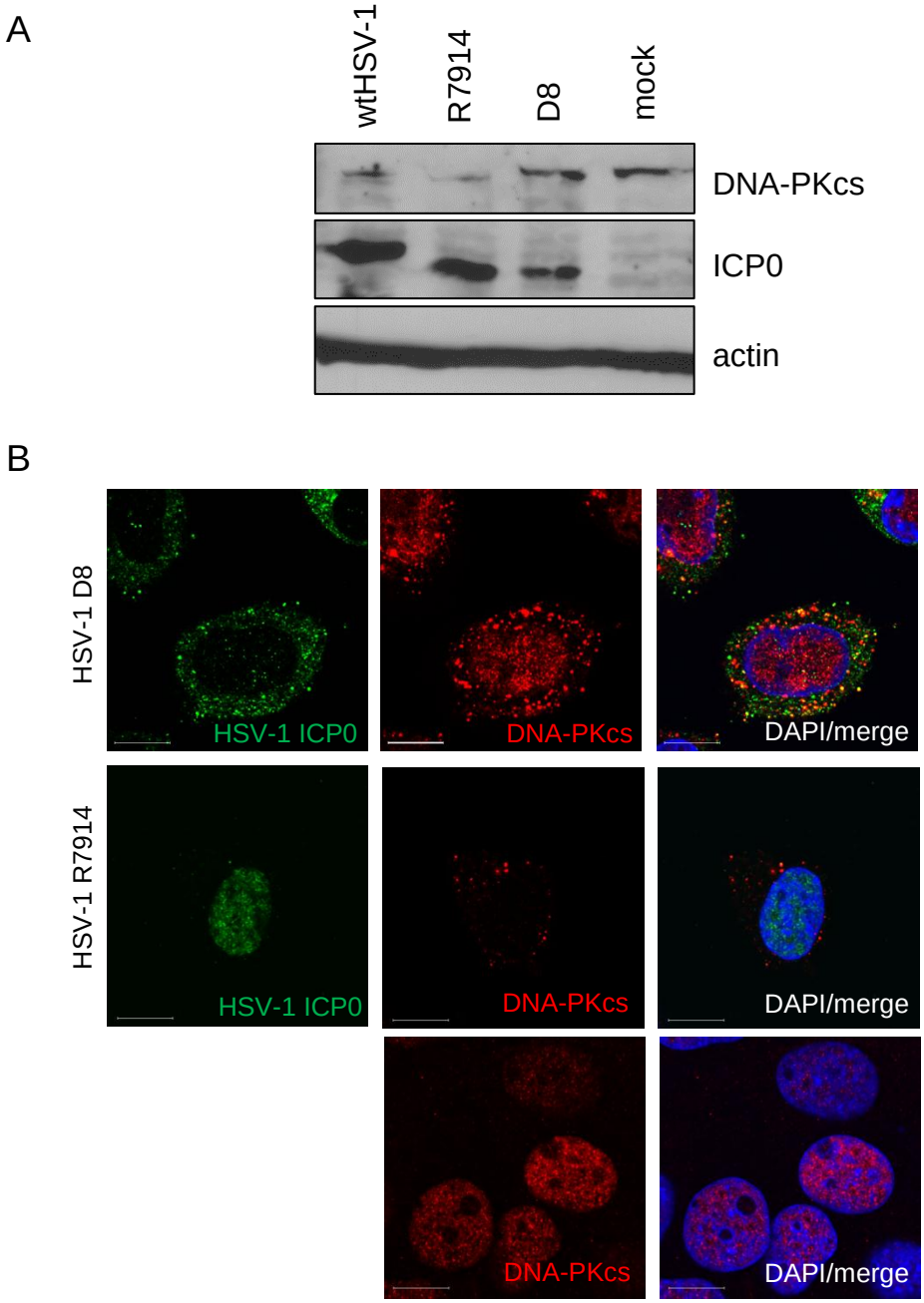



**Fig 2.** Localization and degradation of DNA-PKcs in cells infected with nuclear or cytoplasmic ICP0. **(A)** Western analysis of AT22IJE-T cells at 20h after mock-infection or infection with wtHSV-1 (MOI 3), rHSV-1 D8 (MOI 3) or HSV-1 R7914 (MOI 3). Lysates were processed for Western analysis and stained with antibodies against DNA-PKcs, ICP0, and actin. **(B)** Immunofluorescence analysis of AT22IJE-T cells infected with rHSV-1 D8 (MOI 3) or HSV-1 R7914 (MOI 3) at 20h post infection. Cells were stained with antibodies specific for ICP0 (green) and DNA-PKcs (red) and an FITC-labeled or AF594-labeled secondary antibody, respectively. DAPI was used to stain cell nuclei. Scale bars, 10 μm.

C

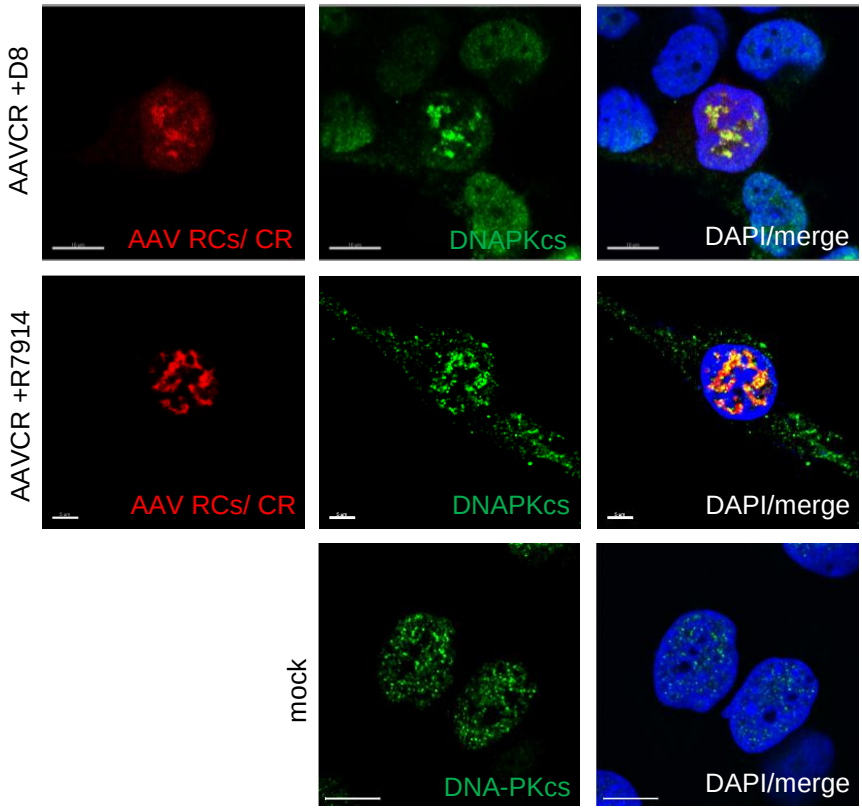



**Fig 2. (C)** Immunofluorescence analysis of AT22IJE-T cells coinfected with rHSV-1 D8 (MOI 1.5) and AAVCR (MOI 500) or HSV-1 R7914 (MIO 1.5) and AAVCR (MOI 500) at 20h post infection. AAVCR replication compartments (RCs) were visualized by binding of the AAVCR-encoded mCherry-Rep 68/78 fusion protein (CR) to AAV DNA (red). Cells were stained with a primary antibody specific for DNA-PKcs and a secondary FITC-labeled antibody (green). DAPI was used to stain cell nuclei. Scale bars, 10 µm.

example, ICP0 does not translocate to the cytoplasm but remains in the nucleus throughout infection (8). Interestingly, in this cell line, HSV-1 is not able to induce degradation of DNA-PKcs (Fig. 1B; (14)). In cells coinfected with AAV2 and HSV-1, we also observed ICP0 mainly in the nucleus of coinfected cells, both early (Fig. 1C i) and late (Fig. 1C ii) during AAV2 replication; although in this fibroblast cell line (AT22IJE-T), ICP0 translocation into the cytoplasm can be observed in cells infected with HSV-1 alone (Fig. 1A and C). Based on these observations we set out to investigate the role of ICP0-localization on degradation of DNA-PKcs in coinfected cells. To study ICP0-localization-dependent degradation of DNA-PKcs, two different HSV-1-ICP0 mutant viruses were used in the following experiments. The ICP0 mutant virus (HSV-1 R7914, strain F (13)) which carries an aspartate-to-alanine substitution in the ICP0 coding sequence at amino acid position D199, is retained in the nucleus (13). In contrast, rHSV-1 D8 (17syn strain, (4)) encodes an ICP0 protein that lacks a nuclear localization signal and, therefore, ICP0 is restricted to the cytosol. Western and immunofluorescence analysis of infected cells revealed that degradation of DNA-PKcs depends solely on nuclear ICP0, without subsequent localization to the cytoplasm (Fig. 2A and B). In cells expressing cytosol restricted ICP0, DNA-PKcs was not degraded (Fig. 2A and B). In cells coinfected with AAVCR (expressing mCherry fused to the Rep 68/78 ORF) and either rHSV-1 D8 or HSV-1 R7914, DNA-PKcs was readily detected within AAV2 RCs (Fig. 2C), supporting pervious results in cells coinfected with AAV2 and wtHSV-1 (see 4.1).

4.3.2.2 Influence of Ku70 on localization and delayed degradation of DNA-PKcs in coinfected cells.

As the results above showed that the interference with HSV-1 induced degradation of DNA-PKcs cannot be explained by AAV2 induced inhibition of ICP0 expression, we set out to examine the expression and subcellular localization of the DNA-PKcs binding protein Ku70 (3). The heterodimers of Ku70 and Ku80 interact with DNA-PKcs to form the DNA-PK complex that is involved in repair of DNA double-strand breaks (DSBs) by NHEJ (3). Ku70 is responsible for initiation of NHEJ by recruiting DNA-PKcs (12) and other NHEJ factors to sites of double-strand breaks (3). Interestingly, immunofluorescence analysis revealed robust recruitment of Ku70 into AAV2 RCs (Fig. 3A), while in cells infected with HSV-1 alone, Ku70 was within HSV-1 RCs and in the surrounding nucleoplasm (Fig. 3A). We hypothesized that Ku70 might be involved in recruitment of DNA-PKcs into AAV2 RCs. Besides its recruiting function, Ku70 might also be directly involved in extending the stability of DNA-PKcs in coinfected cells, by its described function as de-ubiquitinating enzyme (2). Amsel et al showed that Ku70 has a regulatory effect on Bax-mediated apoptosis by decreasing the ubiquitination of Bax and thereby blocking Bax from proteasome-dependent degradation (2).

We first looked at the expression levels of Ku70 in productively infected cells. Western analysis showed no differences in Ku70 levels between mock-infected and HSV-1 and AAV2 coinfected cells (Fig. 3B). As Ku70 has been identified as a component of AAV2 RCs (see above, Fig.1A), we next investigated the role of Ku70 in recruitment of DNA-PKcs into AAV2 RCs. For this, cells were treated with siRNA

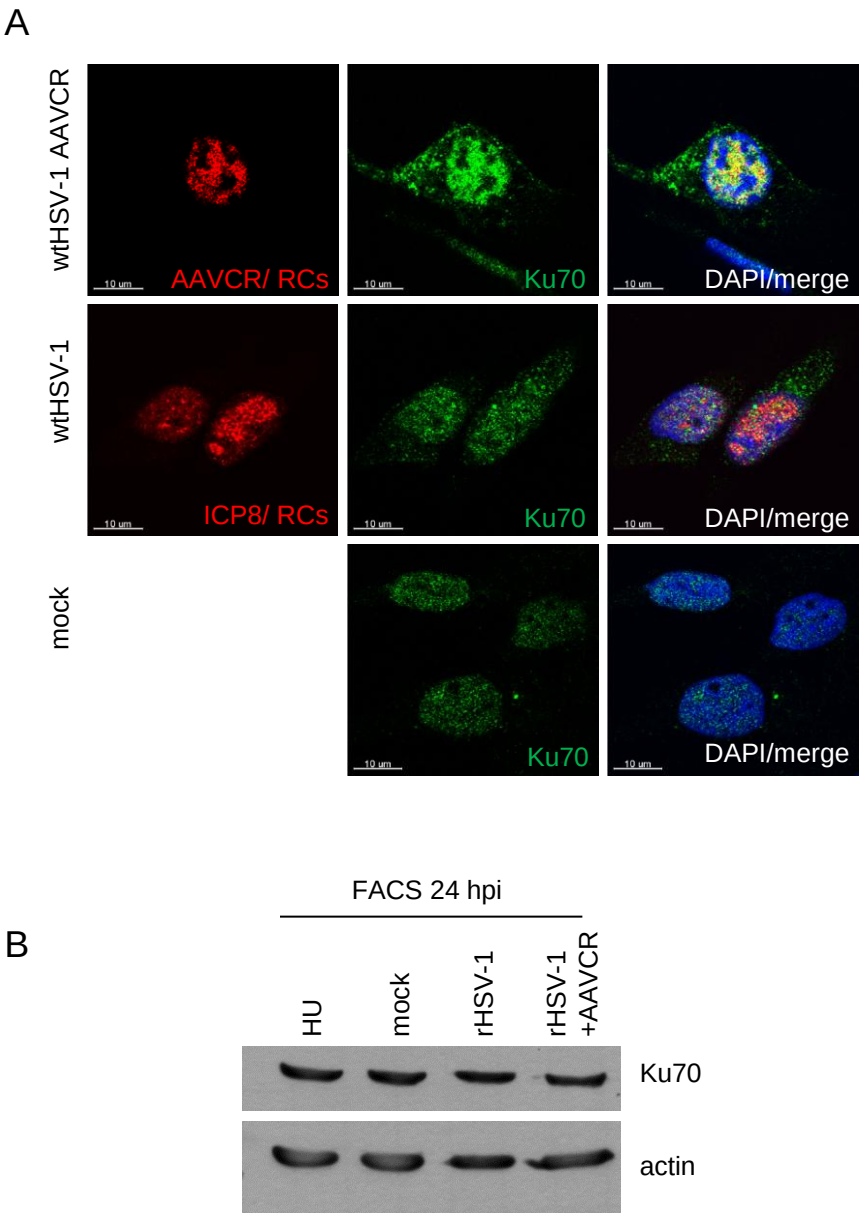



**Fig 3.** Influence of Ku70 on localization and delayed degradation of DNA-PKcs in coinfected cells. **(A and B)** Ku70 expression, localization, and post-transcriptional knock-down in infected cells. **(A)** Immunofluorescence analysis of AT22IJE-T cells infected with wtHSV-1 (MOI 1.5) or coinfected with wtHSV-1 (MOI 1.5) and AAVCR (MOI 500) at 24h post infection. AAVCR RCs (red) were visualized as described in Fig. 2. HSV-1 RCs were visualized with a primary antibody specific for the HSV-1 major DNA binding protein ICP8 and an AF594-labeled secondary antibody (red). Ku70 was detected using a primary antibody specific for Ku70 and a secondary FITC-labeled antibody (green). DAPI was used to stain cell nuclei. Scale bars, 10 µm. **(B)** Western analysis of AT22IJE-T cells sorted (FACS) for productive HSV-1 and AAV2 infection at 24 hpi. Cells were mock-infected, infected with rHSV-1eCFP-ICP4 (rHSV-1; MOI 4), or co-infected (rHSV-1 + rAAV) with rHSV-1eCFP-ICP4 (MOI 4) and AAVCR (MOI 4000). Lysates of sorted cells were processed for Western analysis and stained with the indicated antibodies.

C

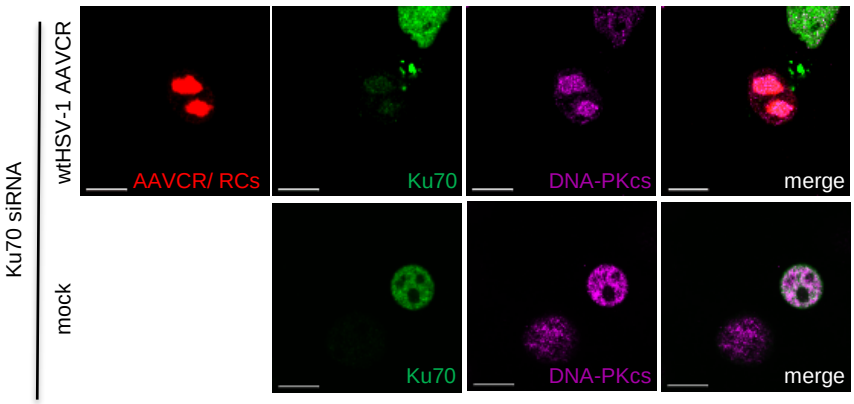



**Fig 3. (C)** Transfection of AT22 IJE-T cells with Ku70 siRNA (Ku70-1/2/3, 20 µM of each) and mock-infected or coinfected with AAVCR (MOI 2000) and HSV-1 (MOI 3) for 24h. AAVCR RCs (red) were visualized as described in Fig. 2. Cells were stained with antibodies against Ku70 (green) and DNA-PKcs (purple) and an FITC-labeled or AF405-labled secondary antibody, respectively. Scale bars, 10 µm.

specific for Ku70 and coinfected with AAV2 and HSV-1. Immunofluorescence analysis showed that DNA-PKcs recruitment into AAV2 RCs and stability (Fig. 3C) did not depend on Ku70.

4.3.2.3 Influence of AAV2 Rep expression on delayed degradation of DNA-PKcs in coinfected cells.

As Ku70 appears not to be involved in stabilization of DNA-PKcs in coinfected cells, we proceeded to investigate the role of the AAV2 Rep proteins in delayed degradation of DNA-PKcs. Western analysis of cells transfected with a plasmid expressing Rep 68/78 from the constitutive cytomegalovirus (CMV) immediate-early promoter/enhancer and infected with HSV-1, demonstrated that overexpression of Rep 68/78 in absence of either AAV2 DNA or AAV2 RCs cannot prevent DNA-PKcs from HSV-1 ICP0-mediated degradation, as neither phosphorylated nor non-phosphorylated DNA-PKcs was detected in Rep-transfected and HSV-1-infected cells (Fig. 4A and B). Similar results were observed in immunofluorescence assays of cells transfected with pRep 78/68 and a plasmid expressing HSV-1-ICP0 (pICP0, Fig. 4C). Independent of the presence or absence of Rep 78/68 expression, ICP0 induced the degradation of DNA-PKcs in transfected cells (Fig. 4C).

4.3.2.4 Influence of AAV2 DNA and AAV2 RCs on delayed degradation of DNA-PKcs in coinfected cells.

Similar to cells coinfected with wtAAV2 and Ad5 (Fig. 5A; (11)), we observed robust phosphorylation of DNA-PKcs at S2056 in cells coinfected with Ad5 and a recombinant, replication-incompetent AAV2 containing the AAV2-ITRs flanking a eGFP coding sequence (rAAVGFP, Fig. 5B). Regarding a previous observation that Ad5 infection alone failed to induce a lucid activation of DNA-PKcs (11), these data suggest that the presence of AAV2 DNA is sufficient to induce vigorous phosphorylation of DNA-PKcs. Therefore, we set out to investigate whether AAV2 DNA alone is sufficient to impede HSV-1 induced degradation of DNA-PKcs, possibly by inducing its phosphorylation. To compare the stabilization of DNA-PKcs in AAV2 and HSV-1 coinfected cells in presence and absence of Rep expression, we performed Western analysis of cells coinfected with HSV-1 and rAAVGFP (no *rep* expression) or coinfected with HSV-1, rAAVGFP, and wtAAV2. Only the cells coinfected with HSV-1, rAAVGFP, and wtAAV2 can support AAV2 DNA replication and the formation of AAV2 RCs, due to the expression of AAV2 *rep* from wtAAV2. Although AAV2 DNA in absence of viral replication was shown to be sufficient to induce robust phosphorylation of DNA-PKcs (Fig. 5B), we observed a strong decrease of DNA-PKcs levels in cells coinfected with rAAVGFP and HSV-1 (Fig. 5C). However, in presence of wtAAV2, DNA-PKcs levels were stabilized (Fig. 5C). These results were confirmed also in immunofluorescence assays of cells coinfected with HSV-1 and either rAAVGFP (encodes eGFP but no Rep) or AAVYR (expressing eYFP fused to the Rep 68/78 ORF, Fig. 5D). Only in cells coinfected with HSV-1 and AAVYR, which supports AAV2 replication, DNA-PKcs was stabilized (Fig 5D), while DNA-PKcs was efficiently degraded in cells coinfected with rAAVGFP (no *rep* expression and therefore no replication) and HSV-1 (Fig 5D).

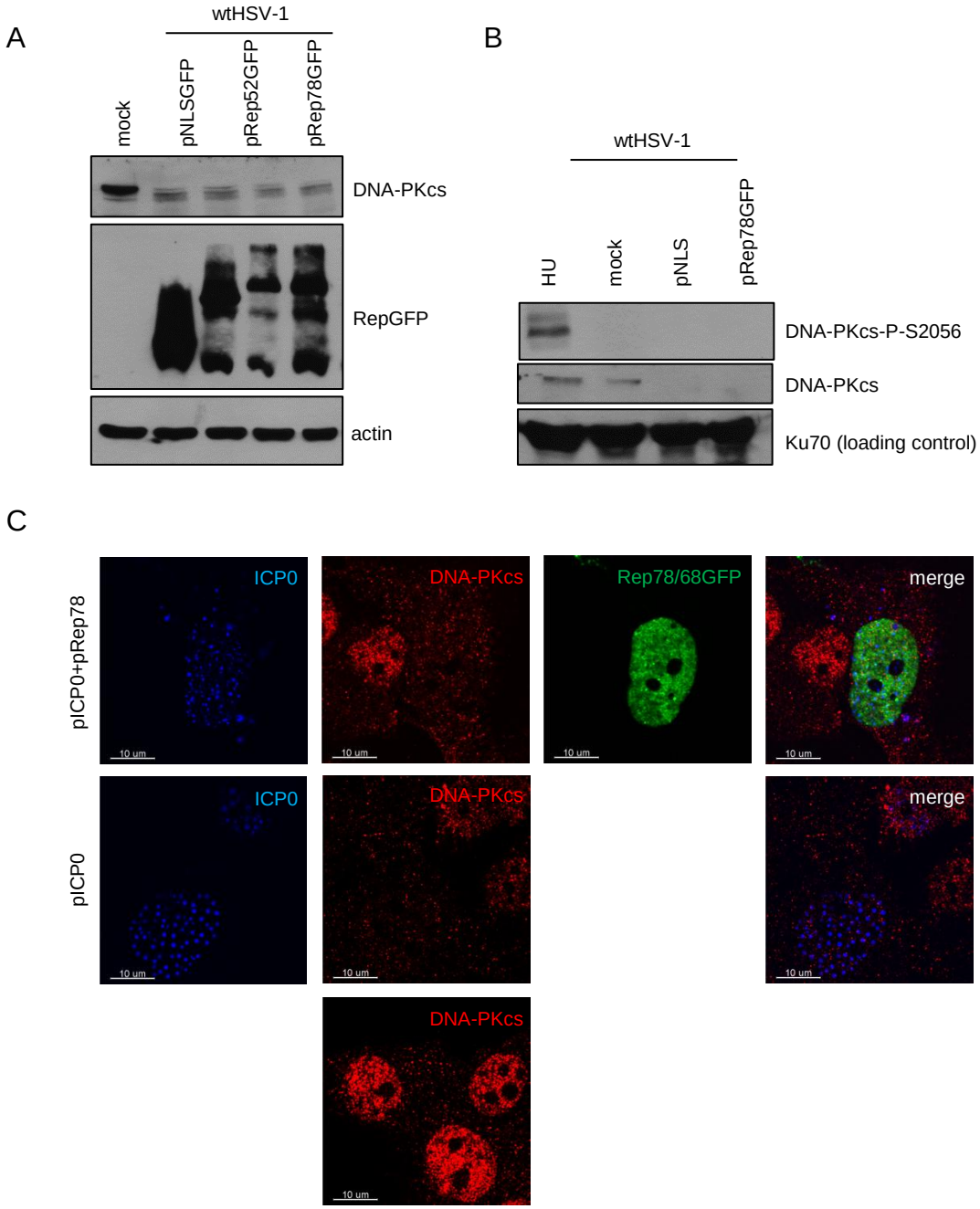



**Fig 4.** Influence of AAV2 *rep* expression on delayed degradation of DNA-PKcs in coinfected cells. **(A and B)** AT22 IJE-T cells were transfected with 0.5 µg of the individual pRepGFP plasmids or the control plasmid pNLSGFP and mock-infected or infected with wtHSV-1 (MOI 3) for 24h. Cells treated with HU (3 mM) served as a DNA-PKcs activation control. Lysates were processed for Western analysis and stained with antibodies against DNA-PKcs, DNA-PKcs-P-Ser2056, GFP, ICP8, Ku70 and actin. **(C)** AT22 IJE-T cells were transfected with 0.1 µg of pICP0 and/or 0.25 µg of pRepGFP. 24h post transfection cells were processed for IF and stained with antibodies against ICP0 (blue) and DNA-PKcs (red) and an AF405-labled or AF594-labeled secondary antibody, respectively. Scale bars, 10 µm.

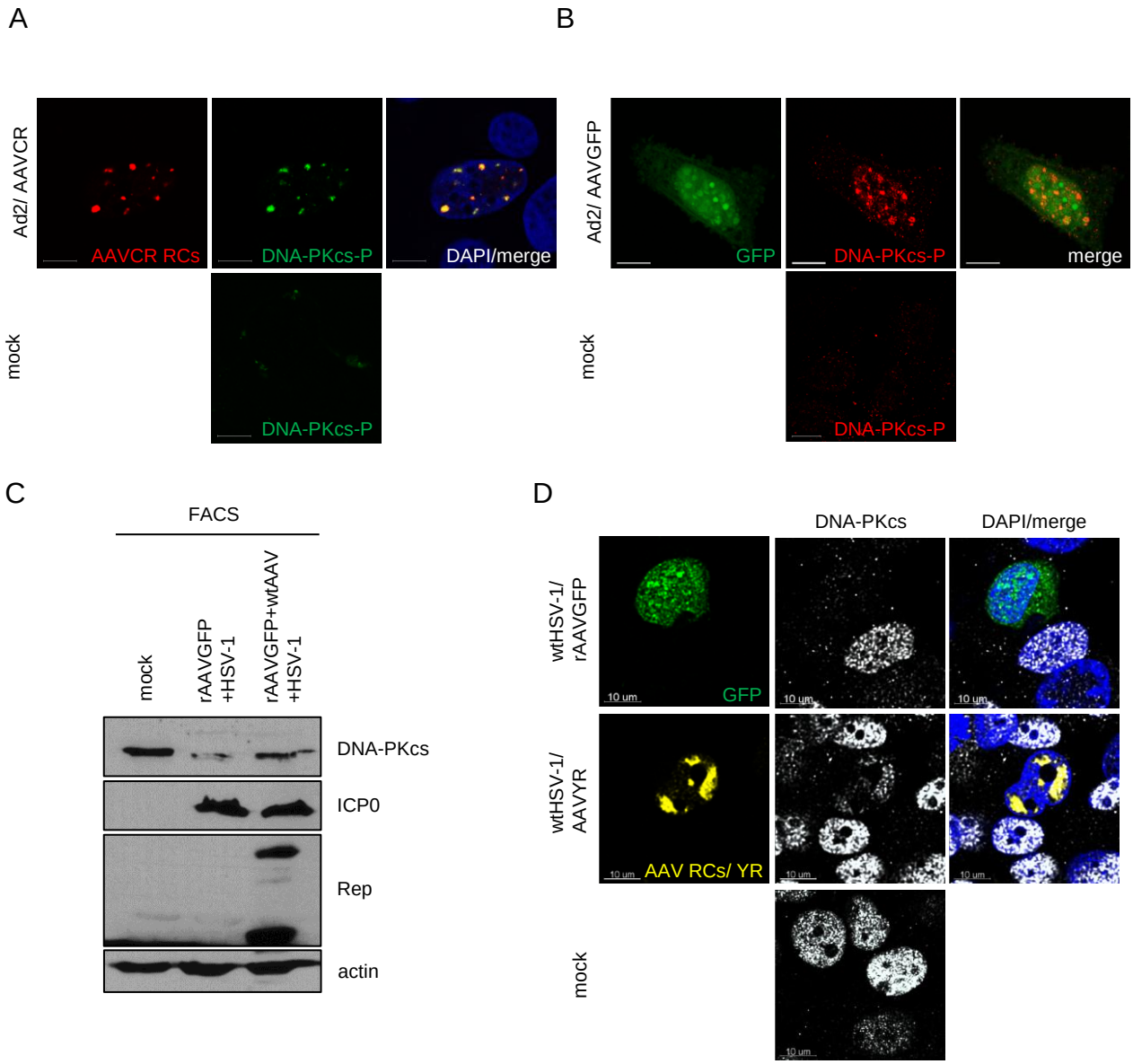



**Fig 5.** Influence of AAV2 DNA and the formation of RCs on delayed degradation of DNA-PKcs in coinfected cells. **(A and B)** Immunofluorescence analysis of AT22IJE-T cells infected with Ad2 (MOI 25) and either AAVCR (MOI 500, A) or rAAV2GFP (MOI 500, B). AAVCR RCs were visualized as described in Figure 2. GFP is expressed constitutively from rAAV2GFP which contains the AAV2 ITR flanking the GFP coding sequence under a CMV promoter. Cells were stained with an antibody against DNA-PKcs and an FITC-labled (green, A) or AF594-labled (red, B) secondary antibody. DAPI was used to stain cell nuclei (A). Scale bar, 10 µm. **(C)** Western analysis of AT22IJE-T cells sorted (FACS) for productive virus infection. Cells were mock-infected, coinfected with wtHSV-1 (MOI 3) and rAAVGFP (MOI 4000), or coinfected with wtHSV-1 (MOI 3), rAAVGFP (MOI 2000), and wtAAV (MOI 2000). Lysates of sorted cells were processed for Western analysis and stained with antibodies against DNA-PKcs, ICP0, Rep, and actin. **(D)** Immunofluorescence analysis of AT22IJE-T cells infected with wtHSV-1 (MOI 1.5) and either AAVYR (MOI 500) or rAAV2GFP (MOI 500). AAVYR RCs were visualized as described in Fig. 1. Expression of GFP see above. Cells were stained with an antibody against DNA-PKcs and an AF594-labled (white) secondary antibody. DAPI was used to stain cell nuclei. Scale bar, 10 µm.

### 4.3.3 Discussion

In order to investigate the question how AAV2 interferes with HSV-1 ICP0-mediated degradation of DNA-PKcs we first explored ICP0 expression levels and a possible role of ICP0-localization in degradation of DNA-PKcs. Experiments with an mutant HSV-1 revealed that degradation of DNA-PKcs depends solely on nuclear ICP0, and no subsequent translocation to the cytoplasm is required. Therefore, although AAV2 influences ICP0-localization in coinfected cells, by supporting retention of ICP0 in the nucleus, delayed degradation of DNA-PKcs in these cells cannot be explained with this observation.

Since Ku70 was observed in AAV2 RCs, we also investigated the role of Ku70 in AAV2 mediated stabilization of DNA-PKcs. Although Ku70 has been described as mediator of DNA-PKcs recruitment to sites of DNA damage (12), during AAV2 replication, recruitment of DNA-PKcs into AAV2 RCs as well as stabilization of DNA-PKcs occurred independently of the presence of Ku70. In a previous study, it has been shown that DNA-PKcs interact with Rep 68/78 in cells coinfected with AAV2 and HSV-1 (9). Therefore, Ku70-independent recruitment of DNA-PKcs into AAV2 RCs may be supported by binding of DNA-PKcs to Rep 68/78 proteins.

Nevertheless, in transfection experiments, Rep 68/78 expression was not sufficient to impede ICP0 mediated degradation of DNA-PKcs. In addition, although AAV2 DNA in absence of viral replication was sufficient to induce robust phosphorylation of DNA-PKcs, delayed degradation of DNA-PKcs was only observed in presence of *rep* expression, DNA replication and the formation of viral RCs, but not in cells coinfected with HSV-1 and a replication-incompetent recombinant AAV2. These data suggests that Rep mediates the recruitment of DNA-PKcs to sites of AAV2 DNA within AAV2 RCs, which further triggers phosphorylation of DNA-PKcs. Then, the AAV2 RC environment impedes HSV-1 ICP0-indcued degradation of DNA-PKcs, resulting in a delayed degradation of DNA-PKcs in cells coinfected with AAV2 and HSV-1.

4.3.4 References (chapter 4.3)


1. **Alazard-Dany, N., A. Nicolas, A. Ploquin, R. Strasser, A. Greco, A. L. Epstein, C. Fraefel, A. Salvetti, and P. O'Hare.** 2009. Definition of Herpes Simplex Virus Type 1 Helper Activities for Adeno-Associated Virus Early Replication Events. PLoS Pathog **5**:e1000340.
2. **Amsel, A. D., M. Rathaus, N. Kronman, and H. Y. Cohen.** 2008. Regulation of the proapoptotic factor Bax by Ku70-dependent deubiquitylation. Proc Natl Acad Sci U S A **105**:5117–5122.
3. **Chapman, J. R., M. R. Taylor, and S. J. Boulton.** 2012. Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. Molecular Cell **47**:497–510.
4. **Everett, R. D.** 1988. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J Mol Biol **202**:87–96.
5. **Glauser, D. L., M. Seyffert, R. Strasser, M. Franchini, A. S. Laimbacher, C. Dresch, A. P. de Oliveira, R. Vogel, H. Buning, A. Salvetti, M. Ackermann, and C. Fraefel.** 2010. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus rep proteins depends on their combined DNA-binding and ATPase/helicase activities. J Virol **84**:3808–3824.
6. **Glauser, D. L., R. Strasser, A. S. Laimbacher, O. Saydam, N. Clement, R. M. Linden, M. Ackermann, and C. Fraefel.** 2007. Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication. molecular mechanisms of interaction. J Virol **81**:4732–4743.
7. **Kawaguchi, Y., R. Bruni, and B. Roizman.** 1997. Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. J Virol **71**:1019–1024.
8. **Lopez, P., C. van Sant, and B. Roizman.** 2001. Requirements for the nuclear-cytoplasmic translocation of infected-cell protein 0 of herpes simplex virus 1. J Virol **75**:3832–3840.
9. **Nash, K., W. Chen, M. Salganik, and N. Muzyczka.** 2008. Identification of Cellular Proteins That Interact with the Adeno-Associated Virus Rep Protein. J Virol **83**:454–469.
10. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol **73**:650–657.
11. **Schwartz, R. A., C. T. Carson, C. Schuberth, and M. D. Weitzman.** 2009. Adeno-Associated Virus Replication Induces a DNA Damage Response Coordinated by DNA-Dependent Protein Kinase. J Virol **83**:6269–6278.
12. **Spagnolo, L., A. Rivera-Calzada, L. H. Pearl, and O. Llorca.** 2006. Three-dimensional structure of the human DNA-PKcs/Ku70/Ku80 complex assembled on DNA and its implications for DNA DSB repair. Mol Cell **22**:511–519.
13. **van Sant, C., Y. Kawaguchi, and B. Roizman.** 1999. A single amino acid substitution in the cyclin D binding domain of the infected cell protein no. 0 abrogates the neuroinvasiveness of herpes simplex virus without affecting its ability to replicate. Proc Natl Acad Sci U S A **96**:8184–8189.
14. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of virology **78**:4783–4796.

# 5 Concluding remarks and perspectives

Productive AAV2 replication, including transcription and DNA synthesis, depends not only on functions provided by the host cell, but also by a helper virus (69). The main goal of this PhD project was to contribute to our understanding of the molecular mechanism of interaction between AAV2 and one of its helper viruses, HSV-1, and the host cell. Similar to other DNA viruses, helper virus supported AAV2 replication takes place in the host cell nucleus in so called RCs (18, 23, 24, 62, 68). Over the past decade, many studies revealed that viruses interfere with the cellular DNA damage sensing and repair machinery (27, 37, 46, 65, 67) and proteins of the DNA-damage response (DDR) pathways have been shown to significantly influence virus replication (27, 37, 46, 65, 67). While some DNA damage sensing and repair proteins support virus replication (35, 41), other factors significantly inhibit the virus and are suggested to belong to the cell's intrinsic antiviral response, which not only affects wt virus infection but also that of viral vectors (15, 36, 64). This work analyzes the localization and activation of DDR proteins upon infection with HSV-1 alone or coinfection with AAV2 and HSV-1, as well as the influence of these proteins on HSV-1 supported AAV2 DNA replication. Since AAV2 is a widely used vector in biomedical applications, the detailed knowledge of the activation and localization pattern of cellular proteins during AAV2 infection, including AAV2 DNA replication and gene expression, may have important practical implications in the field of AAV2 vector research.

One of the central elements of DDR signaling is the MRN complex, composed of Mre11, Rad50, and NBS1 (56). The MRN complex acts as main sensor of ds breaks to further encourage HR or NHEJ processes to repair DNA lesions (32). The MRN complex has been shown to be inhibited during Ad and Ad-supported AAV2 replication (6, 54, 61); therefore DDR signaling in cells coinfected with Ad and AAV2 occurred independent of a functional MRN complex (53). Interestingly, although in HSV-1 infected cells DDR signaling is mediated by MRN and ATM (35), a functional MRN complex is not necessary to mediate DDR downstream signaling to RPA32 in cells coinfected with AAV2 and HSV-1. However, experiments in NBS1 negative cells indicated that the presence of a functional MRN complex or NBS1 clearly aid HSV-1-supported AAV2 DNA replication as well as HSV-1 DNA replication. When comparing the results in Ad (54) and HSV-1 supported AAV2 replication, we hypothesize that the role of the MRN complex in AAV2 replication is facilitated through its differential effect on the helper virus; however, further experiments are necessary to support this.

The three main kinases mediating signal transmission in response to cellular DNA damage include ATM, ATR, and DNA-PK (63). Similar to cells infected with HSV-1 alone (35, 55, 70), activation of ATM and its down-stream targets p53 and Chk2 was observed also during coinfection with AAV2 and HSV-1. However, in contrast to HSV-1 infection which is enhanced by ATM (35), HSV-1 supported AAV2 replication appeared to be inhibited by this kinase. This observation was even more surprising,

considering that activation of the ATM pathway was reported to be beneficial for Ad supported AAV2 replication (11). The opposite effect of ATM on Ad- versus HSV-1-supported AAV2 DNA replication indicates again that the function of cellular proteins in AAV2 replication is at least in part governed by the type of helper virus. The inhibitory effect of ATM-deficiency on HSV-1 replication might support AAV2 replication in coinfected cells, since HSV-1 supported AAV2 replication only occurs in cells in which HSV-1 replication is efficiently repressed (3, 19, 20).

Although a robust ATR-mediated DDR has been observed in cell infected with AAV2 in absence of a helper virus (8, 28), ATR signaling was not observed in cells coinfected with AAV2 and HSV-1 and, similar to previous reports, also not in cells infected with HSV-1 alone (39, 40, 71). The fact that ATR signaling plays a minor role also in Ad- supported AAV2 replication (53) implies an extensive influence of the helper virus on modulation of the cellular DDR signaling pathways in coinfected cells.

By examining the third main kinase in DDR, DNA-PK, we observed HSV-1 ICP0-mediated degradation of DNA-PKcs, which confirmed previous reports (34, 48). This degradation was observed also in cells coinfected with HSV-1 and AAV2, but was markedly delayed (66). The delayed degradation of DNA-PKcs by AAV2 affected the signaling to the downstream targets RPA32, p53, and Chk2 (most evident in ATM-deficient cells). Since DNA-PKcs has been shown to be the primary mediator of DDR in cells coinfected with AAV2 and Ad, we suggest that DNA-PKcs might also play a central role in transmission of DDR signaling in cells coinfected with AAV2 and HSV-1. Overall by comparing our results from cells coinfected with AAV2 and HSV-1 with those of previous studies examining the DDR signaling in cells infected with HSV-1 alone (4, 35, 55, 70), or Ad2 alone (6, 61), or coinfected with AAV2 and Ad2 (11, 53), it is remarkable that although Ad and HSV-1 by themselves induce very different DDRs, the induction and activation patterns of DDR proteins are more similar in the presence of AAV2. Possibly, during AAV2 replication, the cellular DDR is modulated towards DNA-PK-dependent signaling; however, the influence of DNA-PK on AAV2 replication is still under debate and might alter depending on the type of host cell and helper virus. In one report, rAAV2 replication in the presence of HSV-1 or Ad helper functions has been shown to be decreased in absence of DNA-PK (10) while another report showed enhanced wtAAV2 genome replication in absence of DNA-PK activity when Ad was the helper virus (11). In the latter case, it has been suggested that loss of DNA-PK could lead to less circularization of the AAV2 genome which might promote AAV2 DNA replication (11). DNA-PK might also be implicated in self circularization (13, 58), concatemerization (42), and integration (38) of recombinant AAV2 genomes. But analogous to wtAAV2 infection, the data cannot be generalized, since also inhibition of rAAV2 genome integration by DNA-PKcs has been reported (59). On the molecular basis, it is proposed that the hairpin shaped AAV2-ITRs are targeted by cellular NHEJ proteins, including DNA-PK (10, 26), similar to DNA hairpin structures in mammalian cells (emerge during the V(D)J recombination (33)). Independent of its role in NHEJ events, there is preliminary evidence that DNA-PKcs may mediate modification of the large Rep proteins upon AAV2 and Ad coinfection (11). The group of J.P. Trempe showed that phosphorylation of the AAV2 Rep proteins alters their interactions with the AAV2 ITRs (43), thus DNA-PK my play an

essential role in regulating Rep-mediated processes of viral DNA replication (11, 53), including terminal resolution. Besides the effect on AAV2 ITRs, the observed interaction between Rep68/78 and DNA-PKcs (44) may promote recruitment of DNA-PKcs into AAV2 RCs, since Ku70 (mediator of DNA-PKcs recruitment to sites of DNA damage, (60)), is not required for localization of DNA-PKcs in HSV-1-supported AAV2 RCs.

Since modulation of the HSV-1-induced DDR by AAV2 was observed, we set out to investigate the mechanism of delayed degradation of DNA-PKcs in coinfected cells. In line with previous results (21), ICP0 expression levels were not affected by AAV2. In addition, HSV-1-mediated degradation of another target of ICP0, USP7 was not influenced by AAV2. Therefore, we exclude the possibility of an overall inactivation of ICP0-mediated cellular protein-degradation in coinfected cells. Although AAV2 influences ICP0-localization, by supporting retention of ICP0 in the nucleus, delayed degradation of DNA-PKcs in coinfected cells cannot be explained with this observation. In contrast, we could show that degradation of DNA-PKcs by HSV-1 solely requires nuclear localization of ICP0, without translocation to the cytoplasm. As mentioned above, we observed recruitment of DNA-PKcs into late AAV2 RCs in absence of Ku70, indicating that Ku70 is not required for stabilization of DNA-PKcs. We next assessed whether a direct influence of the AAV2 Rep proteins or the AAV2 DNA might support DNA-PKcs stability. Although Rep86/78 has been shown to interact with DNA-PKcs (44), in transfection experiments, Rep68/78 expression was not sufficient to impede ICP0-mediated degradation of DNA-PKcs. Finally, we could also exclude AAV2 DNA as exclusive source of increased stabilization of DNA-PKcs in coinfected cells. Although AAV2 DNA in absence of viral replication was sufficient to induce robust phosphorylation of DNA-PKcs, delayed degradation of DNA-PKcs was only observed in presence of Rep expression, DNA replication, and the formation of viral RCs, but not in cells coinfected with HSV-1 and a replication incompetent rAAV. On the basis of the results above, we hypothesize that Rep68/78 mediates recruitment of DNA-PKcs to sites of AAV2 DNA within AAV2 RCs, which further triggers phosphorylation of DNA-PKcs. Only the formation of AAV2 RCs creates an environment that impedes HSV-1 ICP0-induced degradation of DNA-PKcs, resulting in the observed delayed degradation of DNA-PKcs in coinfected cells. One potential function of DNA-PKcs and down-stream factors within AAV2 RCs might be to trigger the recruitment of DNA damage repair factors. It is suggested that these proteins are involved in AAV2 DNA replication, including initial second-strand synthesis of the AAV2 genome (8, 16, 17, 45, 50). For a detailed discussion see chapter 3.6.

Another potential function of cellular repair proteins in viral replication compartments might be to protect viral DNA from DNA damage caused by viral DNA replication or endogenous DNA damaging agents, such as reactive oxygen species (ROS) in form of superoxide anions and hydrogen peroxides. ROS are produced by a large number of different enzymes (xanthine oxidase, nitric oxide synthetase, p450 cytochromes) and organelles (mitochondria, peroxisomes) as a metabolic byproduct; however, elevated ROS levels are produced by NADPH oxidases (NOX, (5)). These radicals react readily with proteins, lipids, carbohydrates, and nucleic acids (5). Indeed, we

and others observed elevated ROS levels during infection with HSV-1 (2, 29, 30, 52) and coinfection with AAV2 and HSV-1. Since DDR factors are also activated in infected cells in absence of elevated ROS levels, we conclude that viral DNA and/or viral proteins are directly responsible for the activation of DDR signaling in infected cells. Still, it is tempting to speculate that one effect of DNA repair factors within AAV2 and HSV-1 RCs might be to protect viral DNA from ROS induced damage.

The most common types of DNA damage induced by ROS are oxidation of purine or pyrimidine bases as well as DNA SSBs (22) which can be repaired by the cells BER/SSBR machinery. Indeed we found several proteins of the cellular BER/SSBR machinery in HSV-1 and AAV2 RCs. PARP1 and phosphorylated XRCC1 were found in both, AAV2 and HSV-1 RCs. Similar to its role in cellular BER/SSBR (14) PARP1 might mediate recruitment of XRCC1 into viral replication compartments. In addition, RCF1 and ligase I were found in AAV2 RCs, supporting a role of the BER/SSBR machinery in AAV2 replication. Besides repair of DNA lesions caused by ROS, the BER/SSBR machinery might be involved in terminal resolution of the ITR, after Rep78 induced nicking at the TRS. Further experiments are necessary to test this hypothesis.

In conclusion, we identified several factors of the cellular sensing and repair machinery as components of HSV-1 and/or HSV-1 supported AAV2 RCs. In addition we investigated the influence of two main players of the cellular DDR (NBS1 and ATM) on AAV2 DNA replication. The data contribute to a better understanding of the complex mechanisms of interaction between AAV2, its helper viruses, and the coinfected cell.

Since induction of a cellular DDR can mediate a cell cycle arrest via checkpoint activation (72), it will be interesting to investigate the induction of a cell cycle arrest in cells coinfected with AAV2 and HSV-1, and to assess whether the observed modulation of the cellular DDR by AAV2 might result in a differential cell cycle arrest upon infection with HSV-1 or coinfection with AAV2 and HSV-1.

An interesting other future project to further define the function of identified cellular proteins in AAV2 replication could be to systematically identify cellular factors that are not only components of AAV2 RCs, but essential for AAV2 gene expression and viral DNA replication, by small interfering (si)RNA screens. Such knockdown experiments may also identify cellular proteins of AAV2 RCs which, although not preventing AAV2 replication, have an inhibitory effect. The identification of cellular inhibitors and enhancers of helper virus supported AAV2 replication will further contribute to a better understanding of the complex mechanisms of interaction between AAV2, its helper viruses, and the coinfected cell. Moreover, as AAV2 is a widely used gene therapy vector, the detailed knowledge of the function of cellular proteins in AAV2 gene expression and DNA replication will help to improve vector production and transduction efficiency.

It has been shown previously that the HSV-1 major single strand DNA binding protein ICP8 binds to replicating HSV-1 DNA and therefore accumulates in HSV-1 RCs (49). The AAV2 Rep78/68 proteins contain also a DNA-binding motive which allows site-specific binding of Rep78/68 to AAV2 DNA as well as its accumulation in AAV2 RCs (9, 18, 20, 25, 47, 57). In order to determine the localization of DNA damage sensing

and repair proteins during HSV-1 replication or HSV-1 supported AAV2 replication, in the present study cellular proteins were co-visualized with HSV-1 ICP8 or AAV2 Rep78/68 protein, respectively. However, it is important to note that due to limitations in the resolution of Confocal Laser Scanning Microscopy, we cannot make any conclusion concerning the co-localization on the protein level. In theory, the resolution is limited to about half of the wavelength of the light. In our experiments, fluorescent dyes with a wavelength in the range of 400-600nm were used for imaging. To determine whether a specific cellular protein of interest is indeed recruited into AAV2 or HSV-1 RCs, independent of its direct colocalization with AAV2 Rep78/68 or HSV-1 ICP8, we assessed the localization of that particular cellular protein in the three-dimensional space (data not shown).

Altogether, the identification of cellular DNA sensing and repair proteins in HSV-1 supported AAV2 RCs indicates that AAV2 may replicate by using several of these cellular proteins including NBS1, RPA, ATM, or DNA-PK. These findings provide a basis for further examining how DDR proteins affect helper virus supported AAV2 replication. By comparing our results in cells coinfected with AAV2 and HSV-1 with those of previous studies examining the DDR signaling in cells coinfected with AAV2 and Ad5 (11, 53), we can hypothesize on how AAV2 adapts its replication strategy to the nuclear environment manipulated by the helper virus.

The present study was focused on the composition of viral RCs during AAV2 DNA replication in cells simultaneously infected with AAV2 and HSV-1. In absence of a helper virus, AAV2 establishes latent infection by the formation of stable episomes or by integrating its genome into the host chromosome (31, 51). In presence of HSV-1, reactivation from latency can occur, leading to the excision of the AAV2 DNA and viral genome replication (69). Both, insertion and excision of the viral genome may recruit and activate a certain set of DNA damage repair proteins. It is very likely that these proteins are distinct form those detected in AAV2 RCs during viral DNA replication. However, there is indication that proteins of the cellular DDR including DNA-PKcs, affect also AAV2 integration events (7, 12, 59). In further experiments, it would be interesting to examine the influence of DNA sensing and repair proteins on the establishment of latent AAV2 infection as well as reactivation from latency including excision of the viral genomes form the host chromosome. In addition, using a HeLa cell clone (HeLaAAVtCR, (1)) that contains the AAV2 genome integrated into the cellular genome would allow examining the composition of cellular DDR proteins of AAV2 RCs after helpervirus mediated rescue of the integrated AAV2 genome. In this case the initial recruitment of cellular DDR proteins to the site of AAV2 DNA might be altered due to the ds DNA nature of the rescued AAV2 genome compared to the incoming ss AAV2 DNA in cells simultaneously coinfected with the helper virus.

## 5.1 References (chapter 5)


1. **Alazard-Dany, N., A. Nicolas, A. Ploquin, R. Strasser, A. Greco, A. L. Epstein, C. Fraefel, A. Salvetti, and P. O'Hare.** 2009. Definition of Herpes Simplex Virus Type 1 Helper Activities for Adeno-Associated Virus Early Replication Events. PLoS Pathog **5**:e1000340.
2. **Aubert, M., Z. Chen, R. Lang, C. H. Dang, C. Fowler, D. D. Sloan, and K. R. Jerome.** 2008. The antiapoptotic herpes simplex virus glycoprotein J localizes to multiple cellular organelles and induces reactive oxygen species formation. J Virol **82**:617–629.
3. **Bantel-Schaal, U., and H. Zur Hausen.** 1988. Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. Virology **164**:64–74.
4. **Boutell, C., and R. D. Everett.** 2004. Herpes simplex virus type 1 infection induces the stabilization of p53 in a USP7- and ATM-independent manner. J Virol **78**:8068–8077.
5. **Brieger, K., S. Schiavone, F. J. Miller, JR, and K.-H. Krause.** 2012. Reactive oxygen species: from health to disease. Swiss Med Wkly **142**:w13659.
6. **Carson, C., R. Schwartz, T. Stracker, C. Lilley, D. Lee, and M. Weitzman.** 2003. The Mre11 complex is required for ATM activation and the G2/M checkpoint. The EMBO journal **22**:6610–6620.
7. **Cataldi, M. P., and D. M. McCarty.** 2010. Differential effects of DNA double-strand break repair pathways on single-strand and self-complementary adeno-associated virus vector genomes. J Virol **84**:8673–8682.
8. **Cervelli, T., J. A. Palacios, L. Zentilin, M. Mano, R. A. Schwartz, M. D. Weitzman, and M. Giacca.** 2008. Processing of recombinant AAV genomes occurs in specific nuclear structures that overlap with foci of DNA-damage-response proteins. Journal of cell science **121**:349–357.
9. **Chiorini, J. A., L. Yang, B. Safer, and R. M. Kotin.** 1995. Determination of adeno-associated virus Rep68 and Rep78 binding sites by random sequence oligonucleotide selection. J Virol **69**:7334–7338.
10. **Choi, Y. K., K. Nash, B. J. Byrne, N. Muzyczka, and S. Song.** 2010. The effect of DNA-dependent protein kinase on adeno-associated virus replication. PloS one **5**:e15073.
11. **Collaco, R. F., J. M. Bevington, V. Bhrigu, V. Kalman-Maltese, and J. P. Trempe.** 2009. Adeno-associated virus and adenovirus coinfection induces a cellular DNA damage and repair response via redundant phosphatidylinositol 3-like kinase pathways. Virology **392**:24–33.
12. **Daya, S., N. Cortez, and K. I. Berns.** 2009. Adeno-associated virus site-specific integration is mediated by proteins of the nonhomologous end-joining pathway. J Virol **83**:11655–11664.
13. **Duan, D., Y. Yue, and J. F. Engelhardt.** 2003. Consequences of DNA-Dependent Protein Kinase Catalytic Subunit Deficiency on Recombinant Adeno-Associated Virus Genome Circularization and Heterodimerization in Muscle Tissue. J Virol **77**:4751–4759.
14. **El-Khamisy, S. F., M. Masutani, H. Suzuki, and K. W. Caldecott.** 2003. A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage. Nucleic Acids Res **31**:5526–5533.

15. **Everett, R. D., J. Murray, A. Orr, and C. M. Preston.** 2007. Herpes simplex virus type 1 genomes are associated with ND10 nuclear substructures in quiescently infected human fibroblasts. J Virol **81**:10991–11004.
16. **Ferrari, F. K., T. Samulski, T. Shenk, and R. J. Samulski.** 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J Virol **70**:3227–3234.
17. **Fisher, K. J., G. P. Gao, M. D. Weitzman, R. DeMatteo, J. F. Burda, and J. M. Wilson.** 1996. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. J Virol **70**:520–532.
18. **Fraefel, C., A. G. Bittermann, H. Bueler, I. Heid, T. Bachi, and M. Ackermann.** 2004. Spatial and temporal organization of adeno-associated virus DNA replication in live cells. J Virol **78**:389–398.
19. **Glauser, D. L., M. Seyffert, R. Strasser, M. Franchini, A. S. Laimbacher, C. Dresch, A. P. de Oliveira, R. Vogel, H. Buning, A. Salvetti, M. Ackermann, and C. Fraefel.** 2010. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus rep proteins depends on their combined DNA-binding and ATPase/helicase activities. J Virol **84**:3808–3824.
20. **Glauser, D. L., R. Strasser, A. S. Laimbacher, O. Saydam, N. Clement, R. M. Linden, M. Ackermann, and C. Fraefel.** 2007. Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication. molecular mechanisms of interaction. J Virol **81**:4732–4743.
21. **Gong, B., Q. Chen, B. Endlich, S. Mazumder, and A. Almasan.** 1999. Ionizing radiation-induced, Bax-mediated cell death is dependent on activation of cysteine and serine proteases. Cell Growth Differ **10**:491–502.
22. **Hegde, M. L., A. K. Mantha, T. K. Hazra, K. K. Bhakat, S. Mitra, and B. Szczesny.** 2012. Oxidative genome damage and its repair: Implications in aging and neurodegenerative diseases. Mechanisms of Ageing and Development **133**:157–168.
23. **Heilbronn, R., M. Engstler, S. Weger, A. Krahn, C. Schetter, and M. Boshart.** 2003. ssDNA-dependent colocalization of adeno-associated virus Rep and herpes simplex virus ICP8 in nuclear replication domains. Nucleic acids research **31**:6206–6213.
24. **Hunter, L. A., and R. J. Samulski.** 1992. Colocalization of adeno-associated virus Rep and capsid proteins in the nuclei of infected cells. J Virol **66**:317–324.
25. **Im, D. S., and N. Muzyczka.** 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell **61**:447–457.
26. **Inagaki, K., C. Ma, T. A. Storm, M. A. Kay, and H. Nakai.** 2007. The Role of DNA-PKcs and Artemis in Opening Viral DNA Hairpin Termini in Various Tissues in Mice. J Virol **81**:11304–11321.
27. **Jiang, M., and M. J. Imperiale.** 2012. Design stars: how small DNA viruses remodel the host nucleus. Future Virology **7**:445–459.
28. **Jurvansuu, J., K. Raj, A. Stasiak, and P. Beard.** 2004. Viral Transport of DNA Damage That Mimics a Stalled Replication Fork. J Virol **79**:569–580.
29. **Kavouras, J. H., E. Prandovszky, K. Valyi-Nagy, S. K. Kovacs, V. Tiwari, M. Kovacs, D. Shukla, and T. Valyi-Nagy.** 2007. Herpes simplex virus type 1 infection induces oxidative stress and the release of bioactive lipid peroxidation by-products in mouse P19N neural cell cultures. Journal of neurovirology **13**:416–425.
30. **Kim, J. C., S. H. Choi, J. K. Kim, Y. Kim, H. J. Kim, J. S. Im, S. Y. Lee, J. M. Choi, H. M. Lee, and J. K. Ahn.** 2008. [Herpes simplex virus type 1 ICP27

induces apoptotic cell death by increasing intracellular reactive oxygen species]. Molekuliarnaia biologiia **42**:470–477.
31. **Kotin, R., M. Siniscalco, R. Samulski, X. Zhu, L. Hunter, C. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns.** 1990. Site-specific integration by adeno-associated virus. Proc Natl Acad Sci U S A **87**:2211–2215.
32. **Lavin, M. F., and S. Kozlov.** 2007. ATM activation and DNA damage response. Cell Cycle **6**:931–942.
33. **Le Deist, F., C. Poinsignon, D. Moshous, A. Fischer, and J.-P. de Villartay.** 2004. Artemis sheds new light on V(D)J recombination. Immunol Rev **200**:142–155.
34. **Lees-Miller, S. P., K. Sakaguchi, S. J. Ullrich, E. Appella, and C. W. Anderson.** 1992. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. Molecular and cellular biology **12**:5041–5049.
35. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A **102**:5844–5849.
36. **Lilley, C. E., M. S. Chaurushiya, C. Boutell, R. D. Everett, M. D. Weitzman, and J. D. Baines.** 2011. The Intrinsic Antiviral Defense to Incoming HSV-1 Genomes Includes Specific DNA Repair Proteins and Is Counteracted by the Viral Protein ICP0. PLoS Pathog **7**:e1002084.
37. **Lilley, C. E., R. A. Schwartz, and M. D. Weitzman.** 2007. Using or abusing: viruses and the cellular DNA damage response. Trends in Microbiology **15**:119–126.
38. **McCarty, D. M., S. M. Young, and R. J. Samulski.** 2004. Integration of Adeno-Associated Virus (AAV) and Recombinant AAV Vectors. Annu. Rev. Genet. **38**:819–845.
39. **Mohni, K. N., A. R. Dee, S. Smith, A. J. Schumacher, and S. K. Weller.** 2012. Efficient Herpes Simplex Virus 1 Replication Requires Cellular ATR Pathway Proteins. J Virol **87**:531–542.
40. **Mohni, K. N., C. M. Livingston, D. Cortez, and S. K. Weller.** 2010. ATR and ATRIP are recruited to herpes simplex virus type 1 replication compartments even though ATR signaling is disabled. J Virol **84**:12152–12164.
41. **Muylaert, I., and P. Elias.** 2007. Knockdown of DNA Ligase IV/XRCC4 by RNA Interference Inhibits Herpes Simplex Virus Type I DNA Replication. Journal of Biological Chemistry **282**:10865–10872.
42. **Nakai, H., C. E. Thomas, T. A. Storm, S. Fuess, S. Powell, J. F. Wright, and M. A. Kay.** 2002. A limited number of transducible hepatocytes restricts a wide-range linear vector dose response in recombinant adeno-associated virus-mediated liver transduction. J Virol **76**:11343–11349.
43. **Narasimhan, D., R. Collaco, V. Kalman-Maltese, and J. P. Trempe.** 2002. Hyper-phosphorylation of the adeno-associated virus Rep78 protein inhibits terminal repeat binding and helicase activity. Biochim Biophys Acta **1576**:298–305.
44. **Nash, K., W. Chen, M. Salganik, and N. Muzyczka.** 2008. Identification of Cellular Proteins That Interact with the Adeno-Associated Virus Rep Protein. J Virol **83**:454–469.
45. **Nicolas, A., N. Alazard-Dany, C. Biollay, L. Arata, N. Jolinon, L. Kuhn, M. Ferro, S. K. Weller, A. L. Epstein, A. Salvetti, and A. Greco.** 2012. Identification of Rep-Associated Factors in Hsv-1-Induced Aav-2 Replication Compartments. J Virol.

46. **Nikitin, P. A., and M. A. Luftig.** 2011. At a crossroads: human DNA tumor viruses and the host DNA damage response. Future Virology **6**:813–830.
47. **Owens, R. A., J. P. Trempe, N. Chejanovsky, and B. J. Carter.** 1991. Adeno-associated virus rep proteins produced in insect and mammalian expression systems: wild-type and dominant-negative mutant proteins bind to the viral replication origin. Virology **184**:14–22.
48. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol **73**:650–657.
49. **Quinlan, M. P., L. B. Chen, and D. M. Knipe.** 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36**:857–868.
50. **Russell, D. W., I. E. Alexander, and A. D. Miller.** 1995. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. Proc Natl Acad Sci U S A **92**:5719–5723.
51. **Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter.** 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J **10**:3941–3950.
52. **Schachtele, S. J., S. Hu, M. R. Little, and J. R. Lokensgard.** 2010. Herpes simplex virus induces neural oxidative damage via microglial cell Toll-like receptor-2. Journal of neuroinflammation **7**:35.
53. **Schwartz, R. A., C. T. Carson, C. Schuberth, and M. D. Weitzman.** 2009. Adeno-Associated Virus Replication Induces a DNA Damage Response Coordinated by DNA-Dependent Protein Kinase. J Virol **83**:6269–6278.
54. **Schwartz, R. A., J. A. Palacios, G. D. Cassell, S. Adam, M. Giacca, and M. D. Weitzman.** 2007. The Mre11/Rad50/Nbs1 Complex Limits Adeno-Associated Virus Transduction and Replication. J Virol **81**:12936–12945.
55. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. The Journal of biological chemistry **280**:30336–30341.
56. **Shrivastav, M., L. P. de Haro, and J. A. Nickoloff.** 2008. Regulation of DNA double-strand break repair pathway choice. Cell Res **18**:134–147.
57. **Snyder, R. O., D. S. Im, T. Ni, X. Xiao, R. J. Samulski, and N. Muzyczka.** 1993. Features of the adeno-associated virus origin involved in substrate recognition by the viral Rep protein. J Virol **67**:6096–6104.
58. **Song, S., P. J. Laipis, K. I. Berns, and T. R. Flotte.** 2001. Effect of DNA-dependent protein kinase on the molecular fate of the rAAV2 genome in skeletal muscle. Proc Natl Acad Sci U S A **98**:4084–4088.
59. **Song, S., Y. Lu, Y. K. Choi, Y. Han, Q. Tang, G. Zhao, K. I. Berns, and T. R. Flotte.** 2004. DNA-dependent PK inhibits adeno-associated virus DNA integration. Proceedings of the National Academy of Sciences of the United States of America **101**:2112–2116.
60. **Spagnolo, L., A. Rivera-Calzada, L. H. Pearl, and O. Llorca.** 2006. Three-dimensional structure of the human DNA-PKcs/Ku70/Ku80 complex assembled on DNA and its implications for DNA DSB repair. Mol Cell **22**:511–519.
61. **Stracker, T. H., C. T. Carson, and M. D. Weitzman.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature **418**:348–352.

62. **Stracker, T. H., G. D. Cassell, P. Ward, Y.-M. Loo, B. van Breukelen, S. D. Carrington-Lawrence, R. K. Hamatake, P. C. van der Vliet, S. K. Weller, T. Melendy, and M. D. Weitzman.** 2004. The Rep Protein of Adeno-Associated Virus Type 2 Interacts with Single-Stranded DNA-Binding Proteins That Enhance Viral Replication. J Virol **78**:441–453.
63. **Summers, K. C., F. Shen, E. A. Sierra Potchanant, E. A. Phipps, R. J. Hickey, and L. H. Malkas.** 2011. Phosphorylation: The Molecular Switch of Double-Strand Break Repair. International Journal of Proteomics **2011**:1–8.
64. **Tavalai, N., P. Papior, S. Rechter, M. Leis, and T. Stamminger.** 2006. Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J Virol **80**:8006–8018.
65. **Turnell, A. S., and R. J. Grand.** 2012. DNA viruses and the cellular DNA-damage response. Journal of General Virology **93**:2076–2097.
66. **Vogel, R., M. Seyffert, R. Strasser, A. P. de Oliveira, C. Dresch, D. L. Glauser, N. Jolinon, A. Salvetti, M. D. Weitzman, M. Ackermann, and C. Fraefel.** 2011. Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection. J Virol **86**:143–155.
67. **Weitzman, M. D., C. T. Carson, R. A. Schwartz, and C. E. Lilley.** 2004. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) **3**:1165–1173.
68. **Weitzman, M. D., K. J. Fisher, and J. M. Wilson.** 1996. Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. J Virol **70**:1845–1854.
69. **Weitzman, M. D., and R. M. Linden.** 2011. Adeno-Associated Virus Biology, p. 1–23. *In* R. O. Snyder and P. Moullier (ed.), Adeno-Associated Virus. Humana Press. Methods in molecular biology. Humana Press, Totowa, NJ.
70. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of virology **78**:4783–4796.
71. **Wilkinson, D. E., and S. K. Weller.** 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. Journal of cell science **119**:2695–2703.
72. **Zhou, B. B., and S. J. Elledge.** 2000. The DNA damage response: putting checkpoints in perspective. Nature **408**:433–439.

# 6 Acknowledgments

I would like to thank all people who made this work possible.

Many thanks to my supervisor Prof. Dr. Cornel Fraefel and the director of the Institute of Virology Prof. Dr. Mathias Ackermann for sharing their knowledge, critical reading of the thesis, and the opportunity to perform my PhD at the Institute of Virology, University of Zurich. I am grateful for the personal support, scientific guidance, and inspiring discussions by my supervisor.

Many thanks to the members of my thesis committee Prof. Dr. Michael Hottiger and Prof. Dr. Urs Greber for helpful discussions and contributions at the thesis committee as well as critical reading of the thesis.

I thank Prof. Dr. Matthew D. Weitzman for supportive suggestions to the published work, as well as all the other coauthors, Michael Seyffert, Regina Strasser, Anna P. de Oliveira, Christiane Dresch, Daniel L. Glauser, Nelly Jolinon, Anna Salvetti, Mathias Ackermann, and Cornel Fraefel for their important contribution.

I wish to thank all my past and present lab colleagues Andrea Leimbacher, Christiane Dresch, and Sahid Chaudhary for all the support and the pleasant working atmosphere. Special thanks go to Bern Vogt, Anna P. de Oliveira, Bruna de Andrade Pereira, Michael Seyffert, and Andreas Braem for a great time and laboratory support. Thanks to our master student Florian Frischherz for contribution to the project and to all neighboring lab members and the secretaries of our institute.

Finally, I am deeply grateful to my family, especially Marco for moral support during my whole studies and taking care of family needs, to Nika, for making me forget stressful moments during my PhD, to my brother, my parents, and my godfather for all the support during my studies, and lastly to my parents, my parents-in-law, and Simone Willi for the best care they provided for my daughter Nika.

# Curriculum Vitae

## Personal Dates:


| | |
|---|---|
| **Surname:** | Vogel |
| **Forename:** | Rebecca |
| **Postal Address:** | Leemattenstrasse 19<br>5442 Fislisbach<br>Switzerland |
| **Phone:** | +41 56 2091038 |
| **E-mail:** | rvogel@vetvir.uzh.ch |


| | |
|---|---|
| **Date of Birth:** | 28.09.1983 in Kassel, Germany |
| **Sex:** | female |
| **Nationality:** | German |
| **Languages:** | German and English (fluent), French (basic), Spanish (course A1.1) |
| **Marital Status:** | unmarried, 1 child (Nika Flurina Vogel) |

## Referee contacts:

Prof Dr. Cornel Fraefel
Institute of Virology
University of Zürich
Phone: (+41) 044 63 58704
e-mail: cornelf@vetvir.uzh.ch

Prof. Dr. Mathias Ackermann
Institute of Virology
University of Zürich
Phone: (+41) 044 63 58702
e-mail: email@vetvir.uzh.ch

## School Education:

| | |
|---|---|
| **1989-2000** | Primary and Secondary School in Kassel |
| **2000-2003** | Grammar School at Jacob-Grimm Schule in Kassel |
| **2003-2005** | Professional education for Biological technical assistant at at Rheinische Akademie, Cologne, Germany |
| **2005-2007** | 4 semester Biology at University of Wurzburg, Germany |
| **2007-2008** | B.Sc. Biology at University of Zurich, Switzerland |
| **2009-2010** | M.Sc. in Molecular and Cellular Biology at University of Zurich, Switzerland |
| **Since May 2010** | Ph.D. Thesis at the Institute of Virology, University of Zurich, Switzerland |

## Research Experience and Training

| | |
|---|---|
| **2005** | Internship at Landesbetrieb Hessisches Landeslabor, Kassel, Germany |
| **2007** | Student Assistant at the Research Center for Infectious Diseases, Laboratory of Prof. Dr. J. Hammerschmidt, University of Wurzburg, Germany |
| **1010** | Training for production of recombinant Adeno-associated virus at the laboratory of A. Salvetti, Ecole Normale Supérieure de Lyon, France |

## Funding

| | |
|---|---|
| **June 2012** | Travel grant from Life science graduate school PhD program in Microbiology and Immunology (MIM) for the 14th International Parvovirus Workshop in Ithaca, NY, USA |

## Teaching

| | |
|---|---|
| **Since 2010** | BIO322. Cell Biology of Viral Infections, University of Zurich, Switzerland |
| **08.2010-10.2010** | Supervision of M. Sc. student apprentice form University of Berlin, Germnay |
| **06.2012-08.2012** | Supervision of B. Sc. student apprentice form University of Zurich, Switzerland |
| **09.2012-10.2013** | Supervision of M. Sc. student form University of Zurich, Switzerland |

## List of Publications

### Original article in peer reviewed journals

1. *Vogel R., Seyffert M., Strasser R., de Oliveira A.P., Dresch C., Glauser D.L., Jolinon N., Salvetti A., Weitzman M.D., Ackermann M., Fraefel C. Adeno-associated virus type 2 modulates the host DNA damage response induced by herpes simplex virus type 1 during coinfection. J Virol.86:143-55 (2012).*
   *My contribution to this publication was as follows: design of the experiments, main experimental work, including co-immunoprecipitation, Western and immunofluorescence analyses, confocal microscopy, flow cytometry, and writing of manuscript.*

2. *Glauser D.L., Seyffert M., Strasser R., Franchini M., Laimbacher A.S., Dresch C., de Oliveira A.P., Vogel R., Büning H., Salvetti A., Ackermann M., Fraefel C. Inhibition of herpes simplex virus type 1 replication by adeno-associated virus rep proteins depends on their combined DNA binding and ATPase/helicase activities. J Virol. 84:3808-24 (2010).*
   *My contribution to this publication was to provide reagents as well as technical and editorial support.*

**Oral presentations at scientific conferences and seminars**

1. *Vogel R., Seyffert M., Strasser R., de Oliveira A.P., Glauser D.L., Salvetti A., Weitzman M.D., Ackermann M., Fraefel C. Adeno-associated virus coinfection modulates HSV-1 induced DNA damage responses. March 2010, Cells and Virus Seminar, University of Zurich, Zurich, Switzerland.*
2. *Vogel R., Seyffert M., Strasser R., de Oliveira A.P., Glauser D.L., Salvetti A., Weitzman M.D., Ackermann M., Fraefel C. Adeno-associated virus coinfection modulates HSV-1 induced DNA damage responses. XIII Parvovirus Workshop. June 2010 Helsinki, Finland.*
3. *Vogel R., Seyffert M., Vogt B., Ackermann M., Fraefel C. Update on AAV2 Mediated Interference with HSV-1 Induced DNA damage response and the cell cycle arrest. March 1012. Friday Seminar, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.*
4. *Vogel R., Seyffert M., Vogt B., Ackermann M., Fraefel C. AAV2 Mediated Interference with HSV-1-ICP0-Induced Degradation of DNA-PKcs. June 2012 Ithaca, NY, USA.*
   *Update on AAV2 Friday Seminar, Institute of Virology, University of Zurich, Zurich, Switzerland.*
5. *Vogel R., Seyffert M., Bräm A. Vogt B., Ackermann M., and Fraefel C. AAV coinfection manipulates helpervirus-induced cell cycle distribution to enhance replication. 4th Swiss Workshop in Fundamental Virology. Feb. 2013, Thun, Switzerland.*
   *Vogel R., Seyffert M., Bräm A. Vogt B., Ackermann M., and Fraefel C. AAV coinfection manipulates helpervirus-induced cell cycle distribution to enhance replication. Cells and Virus Seminar, Institute of Medical Virology and Institute of Molecular Biology, University of Zurich, Zurich, Switzerland.April 2013.*

**Posters at scientific conferences**

1. *Vogel R., Strasser R., de Oliveira A.P., Ackermann M., Fraefel C. Adeno-associated virus coinfection modulates the HSV-1 induced DNA damage response. 2nd Swiss Workshop in Fundamental Virology. Feb. 2010, Gerzensee, Switzerland.*
2. *Vogel R., Seyffert M., Strasser R., de Oliveira A.P., Dresch C., Glauser D.L., Jolinon N., Salvetti A., Weitzman M.D., Ackermann M., Fraefel C. Degradation of cellular DNA damage response protein DNA-PK depends on nuclear. 3nd Swiss Workshop in Fundamental Virology. Aug. 2011, Thun, Switzerland.*
3. *Vogel R., Seyffert M., Strasser R., de Oliveira A.P., Dresch C., Glauser D.L., Jolinon N., Salvetti A., Weitzman M.D., Ackermann M., Fraefel C. Adeno-Associated Virus Type 2 Modulates the Host DNA Damage Response Induced by Herpes Simplex Virus 1 during Coinfection. 22nd Annual Meeting of the Society for Virology. March 2012, Essen, Germany*
4. *Vogel R., Seyffert M., Bräm A. Vogt B., Ackermann M., and Fraefel C. AAV coinfection manipulates helpervirus-induced cell cycle distribution to enhance replication. 23nd Annual Meeting of the Society for Virology. March 2013, Kiel, Germany.*

Zürich, June 22th 2013